Resolution of enantiomers using cyclodextrins in HPLC, FSCE and NMR

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Summary.

The interaction of a wide range of CD macrocycles with three groups of chiral analytes was examined using FSCE, NMR spectroscopy and several different HPLC methodologies. Novel enantiomeric separations of the analytes were observed in many instances.

Of all the HPLC methods examined, chiral resolution was greatest in the polar organic mode especially with the propranolol analogues. The influence of analyte / CD structure on enantio-resolution was discussed, along with the role played by the relevant inherent chromatographic mechanisms. A unique size dependent, steric repulsive effect was proposed to explain the observed %CRF and k′ values in the polar organic mode. Resolution was generally very poor using CDs as mobile phase additives. The low chromatographic efficiency of several of these methods was suggested as a major factor in their inability to show significant chiral resolution. CD immobilisation onto a solid chromatographic support was found to engender different selectivities of the CDs towards the analytes when compared to the results obtained in the polar organic mode or when using CDs as mobile phase additives.

FSCE was found to provide higher chiral separations than HPLC for nearly all the analytes. CDs which had not provided chiral separation of certain analytes in HPLC, were then seen to do so in FSCE. This was generally linked to the much higher separation efficiencies obtained in the FSCE experiments.

There was no continuous direct relationship between K_f values, FSCE and HPLC analysis times/chiral resolution values and NMR generated analyte chemical shifts/signal splitting, when examining the same set of compounds in the presence of different CDs.

LIST OF ABBREVIATIONS

CD(s)	Cyclodextrin(s)
PGC	Porous Graphitic Carbon
HPLC	High Performance Liquid Chromatography
NMR	Nuclear Magnetic Resonance (Spectroscopy)
MeOH	Methanol
MeCN	Acetonitrile
DMSO	Dimethyl Sulfoxide (*d*-6)
EtOH	Ethanol
FSCE	Free Solution Capillary Electrophoresis
COSY	Correlation Spectroscopy
ROESY	Rotational Nuclear Overhauser Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
NOE	Nuclear Overhauser Effect
TEAA	Triethylammonium acetate
HOAc	Glacial Acetic acid

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Finally, I wish to express my sincere thanks and best wishes to all those people, from so many different parts of the world, who worked in the lab. with me and who provided the real learning experience during my time at Bath.

Dedication.

This work is dedicated to my father and the memory of my late mother, who gave me everything, expected nothing in return, and who deserved so much more.

Michael.

CHAPTER ONE
INTRODUCTION

1.1. Chirality.

Drug stereoisomerism is becoming increasingly recognised as an issue having clinical, research and regulatory implications. However, chirality is also an intricate and fundamental part of life and is not merely confined to the laboratory. Hormones, enzymes, proteins and so on, selectively interact with chemical messengers on a stereochemical basis in nearly all living systems. An understanding of chirality is vital to an understanding of many of the processes which underlie nature itself.

1.1.1. The Discovery of Stereochemistry.

The phenomenon of stereoisomerism was discovered via the isolation of two almost identical substances from the tartars deposited by maturing wine. It was the presence of small hemihedral facets on the crystals of racemic sodium ammonium tartrate that led Pasteur (Pasteur 1848, cited in Armstrong 1984) to the observation that the crystals could be divided into two categories. The major product (+)-tartaric acid, was found to be dextrorotatory to polarized light, whereas the minor product, racemic or paratartaric acid, proved to be optically inactive. Pasteur was able to physically separate these isomers and concluded that the individual molecules of (+) and (-) tartaric acid were structurally 'dissymmetric' i.e. related as non-superimposable mirror image forms. It was van't Hoff and Le Bel, both independently in 1874, (cited in Thorburn Burns 1994) who put forward the stereochemical theory associated with isomerism, based on the concept that carbon (and certain other nuclei) could be associated with four different groups such that it was possible to have two mirror images centered around an asymmetric atom.

1.1.2. Stereochemical Definition and Nomenclature.

The three dimensional shape of an organic molecule depends upon the hybridization state of its constituent carbon atoms. When it proves impossible to superimpose the figure of a molecule onto its mirror image, the molecule is dissymmetric or chiral (from the Greek *cheir* meaning hand). The commonest cause of molecular chirality is the presence of an asymmetric carbon atom (silicon, nitrogen, phosphorous and sulphur atoms are possible alternatives) having four different groups linked to the same atom. There are two possible arrangements of these groups which will give rise to a pair of molecules which are related to one another as object to mirror image, variously described as enantiomers, enantiomorphs or antipodes. Enantiomers have identical chemical properties except toward optically active reagents.

Fischer (cited in Casy 1993a) introduced a convention to designate absolute configuration using the small letters D and L to denote the different isomers. When Bijvoet *et al.* 1951 determined the absolute configuration of a rubidium salt using X-ray crystallography, it transpired that the configuration of D-glucose, arbitrarily assigned by Fischer, was by chance correct. The D/L system is still used for amino acids, glyceraldehyde and related carbohydrates. However it is not applicable to chiral molecules which possess either an asymmetric axis or plane and so Cahn, Ingold and Prelog 1956 introduced a new system based on three consecutive operations:

1. The groups around a centre of chirality are ranked according to a set of sequence rules of which atomic number is the prime consideration.

2. The attached groups, ordered A>B>C>D, are viewed so that D (the lowest priority) is seen pointing backwards, away from the viewer.

3. The other groups are then counted starting from A to C so that if this operation is in a clockwise direction the designation is R (Rectus) or if it follows an anti-clockwise movement, S (Sinister).

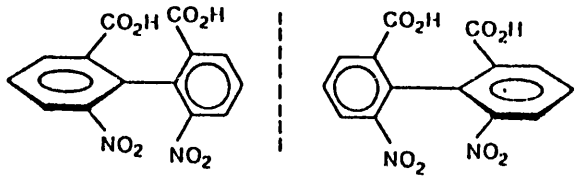
Using this procedure it is possible to systematically designate enantiomers where the existence of chirality is not due to the presence of an asymmetric centre. For example, allenes may be readily derivatised to generate chiral molecules with a chiral axis. The substitution of a hydrogen atom may be sufficient to invoke chirality (Allenmark 1991a). Due to the molecular rigidity of the molecule caused by the double bonds, interconversion between the two forms of the structure is prevented and so stable enantiomers arise.

Hindered rotation around a central bond caused by steric forces may also lead to the formation of stable enantiomers, as exemplified in the case of atropisomers (a form of *conformational* isomers). Certain substituted biaryls may exist as atropisomers due to the steric constraints on rotation engendered by their projecting groups (see Fig. 1.1., p. 5).

Roussel and Favrou 1993 have demonstrated the enantiomeric separation of the atropisomers of some substituted *N*-arylthiazolinethione compounds using γ-CD in HPLC, Mannschreck *et al.* 1984 were able to resolve the atropisomers of methaqualone, a hypnotic agent, using a triacetylcellulose stationary phase in HPLC and Casy 1993 used γ-CD to resolve the atropisomers of telenzepine, a muscarinic antagonist, in NMR.

Steric crowding in a molecule may also give rise to molecular distorsion and hence chirality e.g. helicenes are condensed aromatic hydrocarbons which give rise to right and left handed helical forms (denoted M (-) or P (+)). The energy barrier between these forms is high enough to permit their resolution (Allenmark 1991a).

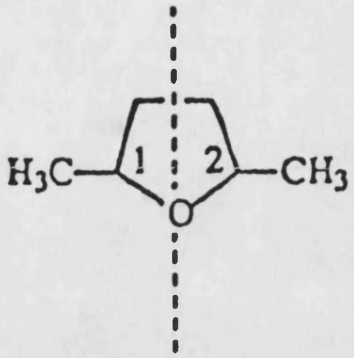
Figure 1.1. Atropisomerism of *o,o'*-dinitrodiphenic acid.



Chiral molecules then, possess either a chiral centre, a chiral axis or planar chirality. However some molecules which contain chiral centres are not themselves optically active. 2,5-dimethyltetrahydrofuran is an example (Fig. 1.2., p.6). The carbon atoms denoted 1 and 2 have four different ligands attached but there is an axis of symmetry within the molecule (dotted line) and therefore the compound's mirror image is superimposable, rendering it optically inactive. Such a molecule is known as a meso compound.

Diastereoisomers or diastereomers are stereoisomers which are not enantiomers of each other. They may arise when a molecule has more than one stereogenic centre e.g. a molecule with two asymmetric carbons could have N (number of optical forms) = 2^n stereoisomers, where *n* is the number of chiral sites. Isomers among the group which are not related as object to mirror image are described as diastereoisomers. Although a molecule may have only one enantiomer, it may have several diastereoisomers. However, two stereoisomers cannot be both enantiomers and diastereoisomers of each other simultaneously (Ahuja 1991). Diastereoisomers differ in their physical interactions with achiral media and may be separated e.g. on the basis of differences in their solubilities or chromatographic retentions on achiral packings.

Fig. 1.2. 2,5-dimethyltetrahydrofuran, a meso compound.



1.1.3. Implications of Chirality in Living Systems.

Many naturally occurring compounds exist in predominantly one chiral form. This may be due to small energy differences in their stability leading to isomeric enrichment i.e. the formation of one enantiomer is favoured over the other (Kondepudi and Nelson, 1985). For example (-) morphine is obtained from the juice of certain poppies and (+)-digitoxin may be extracted from the leaf of the foxglove plant, *Digitalis purpurea*. In contrast, chemical synthesis in the laboratory generally leads to the production of optically inactive racemic mixtures (an equal ratio of enantiomers whose rotation of plane polarised light exactly cancels one another leaving a beam of polarised light unchanged from its original plane).

A large proportion of the thousands of chemicals in use today are chiral. Although more than 50 % of commercial drugs are chiral, less than half of these are marketed in an enantiomerically pure form and only 10% of synthetic drugs are available as pure enantiomers (Parker 1991). Because of the intrinsically chiral basis of many pharmacological interactions it is vital to assess the impact of an optically active xenobiotic on target organisms. Stereoselectivity in both the action and the metabolic conversion of bioactive chiral agents necessitates a full understanding of their pharmacodynamic and pharmacokinetic properties. Certain non-chiral compounds may

also be converted to chiral ones following metabolic action. These *prochiral* agents may then exhibit differing effects *in vivo* due to their 'acquired' chirality e.g. phenytoin undergoes metabolic *para* hydroxylation of one of its phenyl substituents to yield a chiral metabolite and the antihypertensive agent debrisoquine may be converted to its chiral 4-hydroxy derivative following metabolic conversion (Casy 1993a).

Pharmacokinetics is concerned with the absorption, distribution, metabolism and excretion of xenobiotic compounds whereas pharmacodynamics pertains to the action of such agents on target tissues. Pharmacodynamics comprises the receptor-effector coupling, that sequence of biological and biophysical events which is initiated by the receptor activation and leads to the end effect. The interaction is based on a chemical and thereby implicitly steric process between e.g. drugs and their specific receptor sites. Enantiomers may differ not only in their activity but also in their action and may even behave anatgonistically e.g. (-)-isopropylnoradrenaline acts as an agonist on α_1-adrenergic receptors in rats while the (+)-isomer acts as a competitive antagonist with equivalent affinity (Ariëns 1971).

Russell and Hills 1971 were among the first to demonstrate how enantiomers can induce different sensations *in vivo*. They showed how D-aspartamine tasted sweet while L-aspartamine was tasteless and that 4R-carvone had an odour of caraway while 4S-carvone smelt of spearmint. This difference in our perception of enantiomers is thought to be largely a result of the direct chiral discriminating action by receptor proteins, which means that the chiral binding site of the receptor preferentially binds one of the enantiomers (Allenmark 1990). The ratio of enantiomeric activity is known as the eudismic ratio and the isomers may be termed eutomers or distomers respectively, dependent on whether they produce the desired effect or are in some sense 'inactive', which may be manifested in various ways e.g.:

- The enantiomers may have opposite effects not involving competitive antagonism e.g. in DMBB, a barbiturate, the S(-)-isomer acts as a depressant, the R(+)-isomer as a convulsant and *rac*-DMBB as a convulsant (Büch *et al.* 1973).

- The distomer may antagonise a side-effect of the eutomer. (+)-indacrinone is a diuretic and creates a side-effect of uric acid retention which is antagonised by the (-)-isomer, a uricosuric (Tolbert *et al.* 1981).

- The enantiomers may produce different effects each of which may be of therapeutic value e.g. davron is 2R,3S-(+)-dextropropoxyphene, an analgesic, whereas norvad (note the inversion of the trade names!) is 2S,3R-(-)-levopropoxyphene its enantiomer, which acts as an antitussive (Drayer 1986).

If a chiral agent can be optically resolved there is the possibility of studying the behaviour of its enantiomers *in vivo* and therefore to generate the necessary data for its market approval. EU guidelines (CPMP, 1988) state that in the case of a racemic drug already on the market, any application for marketing one of the enantiomers must be accompanied by full documentation, thus regarding it as a completely new substance. The FDA already requires information on stereochemical drug development (Fassihi 1993). If such information had been required in the late 1960's, it may have helped to prevent the thalidomide disaster. It has been reported that the teratogenic activity of thalidomide may reside exclusively in the S-isomer (Blaschke *et al.* 1979).

The different behaviour of enantiomers in biological systems has created a demand for analytical methods to determine the enantiomeric composition or purity of xenobiotics or their metabolites. Metabolism of enantiomeric drugs is exemplified in the case of propranolol a beta-adrenergic blocking agent (which has been examined in this thesis in sections 3.3., 4.3. and 5.3.). The (-)-isomer is 100-times more effective than the (+)-isomer and the oral clearance rate of the (+)-isomer is 40-50% higher (Coltart and Shand

1970 and Lindner *et al.* 1989). Other examples of chiral agents with clear differences in affinity and intrinsic activity are the adrenergic agents, some of which have also been examined in this thesis (the phenethylamines in sections 3.2., 4.2. and 5.2.). Table 1.1. (Allenmark 1991a) shows the ratios of (-)/(+) enantiomers for two phenethylamines related to their intrinsic activity (maximal effect as fraction of that of (-)-isoprenaline) and pD_2 value (negative log of the concentration producing 50% of the maximal effect of the compound).

Table 1.1. Ratios (-)/(+) of enantiomers for pD_2 values and intrinsic activity. See text for explanation of parameters.

Compound	(-)/(+) pD_2	(-)/(+) intrinsic activity
Isoprenaline		
β_1-receptors	8.45/7.42	1.00/0.96
β_2-receptors	7.42/5.87	1.00/1.04
Salbutamol		
β_1-receptors	6.17/4.47	0.51/0.56
β_2-receptors	6.20/3.75	1.00/0.96

1.2. Cyclodextrins (CDs)- Structure and Properties.

The analytical methods used in this thesis have employed CDs as the chiral selector for different groups of optically active compounds. Many other chiral agents besides CDs have been investigated for their ability to provide enantiomeric resolutions. Section 1.3. details some of these chiral selectors and the different techniques in which they have been utilised.

As early as 1908 Schardinger discovered that new crystalline carbohydrates could be formed during the degradation of starch by the micro-organism *Bacillus macerans*

(Allenmark 1991b). These substances were dubbed Schardinger dextrins and are now commonly known as cyclodextrins (CDs). The three most common and naturally occurring CDs are α-, β- and γ-CD with 6, 7, and 8 α-D-(+)-glucose units respectively, joined together by α-(1,4) linkages forming a hollow truncated cone with an axial cavity (see Fig. 1.3.). CDs with more than 8 glucose units have been reported but their stability is low (Duchêne *et al.* 1987). The external face of each macrocycle is marked by hydroxyl groups found on either the wider secondary opening in the 2 and 3 positions or on the narrower primary opening in the 6 position, making the exterior hydrophilic in character. The internal face of the CDs is by contrast relatively hydrophobic due to the presence of the glucosidic oxygen atoms. Table 1.2. gives some physico-chemical properties of the three parent CDs.

Figure 1.3. Schematic representation of CD molecule. See Table 1.2. for details.

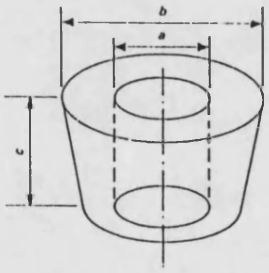


Table 1.2. Physico-chemical properties of α-, β- and γ-CD. See figure 1.3. above.

CD	Dimensions Å			Cavity volume Å^3	Molecular mass	Specific optical rotation $[\alpha]_D25$	Solubility in water at 25°C (g/100 ml)
	a	b	c				
α	5.7	13.7	7.8	174	972	+ 150	14.5
β	7.8	15.3	7.8	262	1135	+ 162	1.8
γ	9.5	16.9	7.8	427	1297	+ 177	23.2

The low aqueous solubility of β-CD has been attributed to its formation of aggregates having a herring-bone like structure, which reduces the ability of the macrocycle to hydrogen-bond with water molecules and hence decreases its solubility (Coleman *et al.* 1992). The aqueous solubility of β-CD depends on the nature and concentration of any added organic modifier. MeCN produces the greatest increase in solubility, up to 20 % v/v, while MeOH steadily reduces β-CD and γ-CD solubility to <0.7 g/100 ml at 20 % v/v (Taghvaei and Stewart 1991 and Chatjigakis *et al.* 1993). Urea and NaOH have also been shown to alter the solubility of α-, β- and γ-CD (Pharr *et al.* 1989).

1.2.1. Modified CDs.

Most CD derivatives are highly water soluble, generally more so than the parent CD from which they are produced. Methyl CDs are available with varying degrees of substitution around the CD rim e.g. either all C2 and C6 groups are methylated to give the dimethyl CDs or all the hydroxyls are methylated (C2, C3 and C6) to give the trimethyl CDs, with water solubilities of 57 and 31 g/100 ml respectively. However one disadvantage is their decrease in solubility with temperature. The hydroxypropyl CDs are also highly water soluble, >50 g/100 ml, but it is impossible to selectively substitute the CD hydroxyl groups as the hydroxyl reactivity changes as the derivatization reaction proceeds (Duchéne and Wouessidjewe 1990). The result is a mixture of CD derivatives. Substitution of CDs at the hydroxy groups at the 3-position of the glucose unit is well known to be more difficult than substitution at the 2- and 6-positions (Deege *et al.* 1993). Unlike the methyl derivatives however, the solubility of these CDs does not decrease with temperature as their dissolution is endothermic. Hydroxethyl CDs closely resemble the hydroxypropyl derivatives and have comparable water solubilities, 50 g/100 ml, (Konishi *et al.* 1994).

Many other CD derivatives have been produced and the application of some of them to the field of enantio-separations have been described in sections 3.1.1., 4.1.6. and 5.1.6.

1.2.2. Basis of Stereochemical Interaction with CDs.

Dagleish 1952, who was examining the chiral separation of aromatic amino acids using paper chromatography, is credited with first having considered the three-point interaction concept in chromatographic separation. In order for either of two enantiomers ('guest' molecules) to have a stronger interaction with a chiral selector (the 'host'), it is necessary that they interact with the chiral selector in such a way that of three possible points of interaction between them, only one enantiomer can fulfill all three i.e. at least one of the interactions is stereochemically based. This results in its preferential interaction (in the case of Dagleish this was evidenced by a stronger retention of one enantiomer on the chiral cellulose adsorbant) which may allow for the physical separation of the enantiomers. The forces which produce these host-guest interactions may be repulsive as well as attractive (Davankov and Kurganov 1983). When the sum total of all possible interactions are accounted for, there must be a slight difference (a few calories) in the association of one enantiomer over the other if separation is to be achieved (Boehm *et al.* 1988).

CDs are themselves chiral e.g. β-CD has 35 chiral centres and so they are able to interact with other chiral compounds under stereospecific conditions. Street 1987 has shown that the polarity of CD cavities is similar to that of *n*-octanol. As a result of this apolar cavity CDs can include other apolar/non-polar molecules of appropriate dimensions by virtue of hydrophobic interactions. No bonds are involved in the complex formation which is driven by hydrophobic forces when the CD and its guest are present in aqueous media. In examining a range of substitued aromatic compounds and common organic solvents in the presence of β-CD, Park and Nah 1994 concluded that the formation of inclusion

complexes was dominated by non-polar (hydrophobic) dispersive interactions between the CD cavity and the solute and that hydrogen-bond formation played only a minor role. They also found that increasing guest dipolarity and hydrogen-bond acceptor basicity decreased the complex stability as the guest was then more likely to hydrogen-bond with water, which is more dipolar and hydrogen-bond acidic than β-CD. Inclusion complex formation may be influenced by other factors such as van der Waals interactions, the release of high energy water from the CD cavity and possibly a change in ring strain upon complexation (Boehm *et al.* 1988 and Krustulovic 1989b).

CDs also possess large dipole moments and therefore electrostatic effects may play an important role in substrate binding and orientation within the CD cavity (Sherrod 1992). Sakurai *et al.* 1990 measured the dipole moment of CDs and found that it increased with increasing ring size in the order α-CD < β-CD < γ-CD and that directionally it ran from the secondary opening to the primary opening at an angle to the cavity axis. If guest molecules also possessed a dipole moment they may be more or less likely to interact with the CD depending on the direction of the dipole moment and its strength. This has been supported by other work e.g. Yoshida and Hayashi 1994 found that certain azo guest molecules could complex with α-CD dependant on the nature of the charged group on the guest, which influenced the orientation and/or direction of inclusion complexation.

Under reversed phase conditions hydrogen-bonding between the hydroxyl groups of CDs and other polar groups of guest molecules e.g. amines and hydroxyls, coupled with hydrophobic interactions, is believed to be the cause of enantioselection. These multiple interactions provide the basis of the three-point interaction model mentioned previously. A two point interaction between the CD and guest, such as an electrostatic attraction and a hydrogen bond, might be sufficient for chromatographic separation due to differential adsorption of the transient diastereomeric adducts on the achiral stationary phase (Martens and Bhushan 1992) providing the necessary third point of interaction. Furthermore, if one of the interactions defines an axis rather than a point e.g.

interactions between two aromatic rings, only two interactions may be necessary for chiral resolution (Krstulovic 1988). Section 3.1.5. details the equilibria involved in CD modified reversed phase chromatography.

Under 'polar organic' conditions (see section 3.1.3.) the mode of analyte:CD interaction is less well understood. Inclusion complexation is not thought to occur due to the high amount of organic modifier in the mobile phase which competes with the guest molecule for inclusion into the CD cavity (Chang *et al.* 1986). Various models of the analyte:CD interaction have been proposed, where the analyte sits like a lid over the CD cavity and interacts via hydrogen-bonding with the external hydroxyl groups of the CD (Armstrong *et al.* 1992 and Zukowski *et al.* 1993).

1.2.3. Uses of CDs.

CDs have numerous applications in many scientific fields and industries, especially in the pharmaceutical and healthcare area. This is influenced by the fact that oral administration of α-, β- or γ-CD does not present any adverse toxicity effects and that long term administration does not cause any significant change in the internal organs. However, their parenteral administration is not recommended due to the possibilty of ulceration and nephrotoxic and haemolytic effects (Szejtli 1987). They are also used extensively in catalysis studies, agrochemicals and the food industry.

CDs have been shown to encapsulate a range of volatile substances so that in cosmetics they are used in deodorants to trap unpleasant body odours (Itaro 1987), to complex excreted fatty acids so reducing acne (Unilever 1981) and in breath fresheners to remove odours such as fish and cigarettes (Asama 1980). They have been used in cooking to decrease the odour of mutton (Nagano 1980), raw fish (Goku 1974) and the smell of long term stored rice is reduced by initially cooking it with β-CD (Takeda 1981). Many gases

can be complexed by α-CD, the smallest natural macrocycle, to form solid crystalline products. Cramer and Henglein (cited in Duchéne and Wouessidjewe 1990) were the first to demonstrate this in the 1950's with e.g. xenon, propane and oxygen. Ethylene, which is important in the ripening process of plants, has been included in CDs and sprayed on crops to increase their ripening and krypton 85 has been included by α-CD for use in car batteries (Akad Wissenschaft 1979).

CDs can also be used to stabilise certain compounds by encapsulating and so protecting them from potentially degrading external conditions e.g. heat, light and oxygen. Mitomycin antibiotics are protected from degradation upon complexation with γ-CD, which protects a labile substituent on the antibiotic by encapsulating that portion of the molecule and shielding it from external interactions (Bekers *et al.* 1991). Oils and perfumes have been encapsulated using CDs and shown to remain stable in a dry form for > 1 year (Harangi and Nanasi 1994), vitamins A, D, E and K are more resistant to oxidation when complexed by β-CD (Szejtli *et al.* 1980b) and pyrethroid insecticides, which are sensitive to UV and oxygen, can be stabilised by inclusion with β-CD (Yamamoto *et al.* 1976). CDs may also improve the solubility of some pharmaceutical compounds such as flurbiprofen, which can be complexed by γ-CD (Otagiri *et al.* 1983). However, it is necessary to have a low stability constant between the drug and the CD as well as an increase in aqueous solubility of the complex, to improve the bioavailability of the drug. Sometimes the increase in bioavailability is such that it is possible to propose a decrease in the dose administered (Uekama *et al.* 1983).

1.3. Enantiomeric Separations.

There are a wide variety of analytical methods employing chiral selectors other than CDs, which have been used to separate, quantify and identify enantiomeric compounds, as described in the following sections.

1.3.1. Enantiomeric Separations in HPLC.

Separations may be performed either by using chiral stationary phases or by dissolving optically active reagents in the mobile phase and employing achiral column packings. There are a number of synthetic chiral stationary phases in HPLC based on hydrogen-bonding, π-π interactions, dipole stacking and electrostatic attractions. These so called 'Pirkle phases' (after their inventor William Pirkle) are prepared by reacting the α-amino group of e.g. (R)-phenylglycine with 3,5-dinitrobenzoyl chloride and attaching the product to aminopropyl functionalised silica (Pirkle *et al.* 1984). They have been used to separate the enantiomers of a number of compounds e.g. Wainer *et al.* 1984 separated the enantiomers of α-methylarylacetic acid anti-inflammatory drugs, Nicoll-Griffith 1987 resolved the enantiomers of ibuprofen and Lienne *et al.* 1989 resolved the enantiomers of albendazole sulfoxide, an anthelmintic drug. The only problem with these types of stationary phases is that analytes frequently require derivatization (Krstulovic 1988).

Chiral ions may be added to the mobile phase for the optical separation of analytes in HPLC. Pettersson and No 1983 and Pettersson 1984 were able to separate the enantiomers of chiral carboxylic acids using cinchona alkaloids as chiral counter ions and Pettersson and Schill 1981 used (+)-10-camphorsulphonic acid to resolve some amino-alcohols. Metal complexes have been used in mobile phases creating the technique of chiral ligand exchange. Generally an amino-acid is immobilised on the stationary phase and in the presence of a metal ion dissolved in the mobile phase analytes form diastereomeric complexes of opposite configuration which are then resolved. Davankov pioneered this method in the late 1960's (Krstulovic 1988) and it has been modified by others more recently e.g. Ôi *et al.* 1992 used novel N,S-dioctyl-(D)-penicillamine as a stationary phase with copper (II) ions in the mobile phase to resolve the enantiomers of more than twenty amino-acids.

Wulff and Minarik 1990 used optically active imprinted polymeric sorbents to successfully resolve the enantiomers of α-phenylmannoside. The shape of cavities in the sorbent and the spatial arrangement of interactive groups within these cavities allowed chiral recognition and hence chromatographic resolution to occur. Cellulose based columns such as 'chiralcel OD' can be used in the normal phase chromatographic mode to resolve enantiomers e.g. Shah and Maryanoff 1991 separated the enantiomers and diastereoisomers of pyrroloisoquinoline antidepressants, while Castellani *et al.* 1992 produced novel urea/formaldehyde based gels with copolymerised L-leucinamide to separate a series of organic acid racemates.

Immobilized proteins such as α_1 acid glycoprotein (AGP), bovine serum albumin (BSA) and ovomucoid (OVM) are commonly are employed as chromatographic supports in chiral chromatography. Analytes may be separated as a result of stereospecific binding to certain sites on the protein. Many analytes have been resolved by various workers using AGP columns e.g. Hermansson 1989 (bupivacain and ephedrine), Noctor *et al.* 1990 (oxaminquine) and Schill *et al.* 1986a (cocaine and methadone). BSA has been shown to resolve the enantiomers of ketoprofen (Andersson and Allenmark 1989), oxazepam and benzoin (Erlandsson and Nilsson 1989) and temazepam (Thompson *et al.* 1989). OVM based packings have been used to chirally resolve propranolol (Haginaka *et al.* 1990), chlorprenaline (Miwa *et al.* 1987) and abscisic acid (Okamoto and Nakazawa 1990).

1.3.2. Enantiomeric Separations in Capillary Electrophoresis.

Host-guest complexations have also been carried out with crown ethers using FSCE. These are macrocyclic polyethers containing 1,4-dioxabutan(-O-CH_2-CH_2-O-) units and they are able to form selective complexes with a variety of inorganic and organic cations (Snopek *et al.* 1992). The first reported separation in FSCE was by Kuhn *et al.* 1992a

who separated racemic amino-acids using 18C6-tetracarboxylic acid. The carboxylic acid groups on the crown ethers are thought to affect chiral resolution through the formation of a chiral barrier to analyte entry or perhaps via electrostatic interactions between the host and guest (Kuhn *et al.* 1992b). Snopek *et al.* 1991 also used 18C6-tetracarboxylic acid to effect the enantio-resolution of a group of primary amines e.g. catecholamines, naphthylethylamines and aromatic amino-acids.

Chiral surfactants, which form micelles above certain concentrations, have also been used to resolve chiral compounds e.g. Otsuka and Terabe 1990 used digitonin/sodium dodecylsulphate to separate racemic PTH-amino acids, Dobashi *et al.* 1989 separated N-acetylated amino acid isopropylesters with sodium N-dodecanoyl-L-valinate and Nishi *et al.* 1989 used sodium taurodeoxycholate and sodium cholate to separate chiral binaphthyl derivatives.

Chiral ligand exchange is a method applicable to enantio-separation in capillary electrophoresis. It involves the formation of mixed ternary chelate complexes, consisting of a chiral bifunctional ligand, a transition-metal ion and the chiral analyte. It may be applied to molecules with two polar groups that are at a proper distance from each other so that they act as ligands for the central transition-metal ion (Snopek *et al.* 1992). Gassman *et al.* 1985, Gozel *et al.* 1987 and Gozel and Zare 1988 have used this technique to separate the enantiomers of dansylated amino acids by producing copper(II) ions/L-histidine or aspartame complexes, which then interact with the amino acid derivatives to form diastereoisomeric ternary chelates having different stability constants. This affects their migration order which may then lead to their separation.

Proteins and peptides have also been successfully used as buffer additives in FSCE for the resolution of many chiral compounds. Their mode of interaction is not fully understood as they possess multiple interaction sites which may cause enantio-discrimination via a variety of mechanisms e.g. hydrophobic interactions, hydrogen-

bonding, steric forces etc. Bovine serum albumin has been used for the separation of leukoverin (Barker *et al.* 1992), pindolol, promethazine and warfarin have been examined using a range of protein additives - ovomucoid, bovine serum albumin, cellulase and orosomucoid, (Busch *et al.* 1993), a variety of β-blockers including propranolol have been chirally resolved using cellulase (Valcheva *et al.* 1993) and amino acids and indole lactic acid were optically separated with human serum albumin (Vespalec *et al.* 1993). Birnbaum and Nilsson 1992 used bovine serum albumin cross-linked with gluteraldehyde for the separation of trytophan enantiomers. This technique, called capillary gel affinity electrophoresis, suffers however from the problem of reproducibily preparing narrow gel filled capillary columns. Vancomycin, a peptide and antibiotic agent, has been dissolved in buffers and used as a chiral selector for the separation of dipeptides (Carpenter *et al.* 1992).

1.3.3. Enantiomeric Separations in NMR.

The determination of enantiomeric purity using NMR requires the use of a chiral auxiliary that converts the mixture of enantiomers into a diastereoisomeric mixture. The resonances of diastereoiosmers, of certain nuclei, are anisochronous and thus give rise to different chemical shift values. Chiral lanthanide shift reagents, chiral solvating agents and chiral derivatising reagents are frequently used to enable chiral discrimination in NMR.

Derivatisation of enantiomers is commonly used for chiral purity determination. MPTA, α-methoxy-α-(trifluoromethyl)phenylacetic acid, has been widely used for the NMR examinations of chiral amines and alcohols (Williams *et al.* 1990 and Nieduzak and Carr 1990). Baker *et al.* 1989 used (S)-methyl mandelate to derivatise carboxylic acids and Kato 1990 used a binaphthyl derivative in the presence of 1-methylimidazole to give diastereoisomers of a range of chiral alcohols. Chiral lanthanide reagents form weak

complexes with a large variety of organic compounds and cause their signals to shift (up or downfield) by a magnitude dependant mainly upon the distance between the nuclei of interest and the lanthanide group. Europium, praseodymium and ytterbium complexes have been widely used to induce shifts in a range of chiral sulfoxides, carboxylates and aromatic acids (Guanti 1990). Chiral solvating agents form diastereoisomeric solvation complexes with solute enantiomers via rapid reversible equilibria in competition with the bulk solvent. Non-polar solvents tend to maximise the observed signal shifts while more polar solvents tend to solvate the analyte and any induced chemical shift changes tend to zero (Parker 1991). (R)-2-naphthylethylamine has been used to cause chemical shifts of chiral carboxylic acids (Aitken and Gopal 1990) and 1-(9-anthryl)-2,2,2-trifluoroethanol has been widely employed to examine lactones, ethers and oxaziridines (Pirkle and Adams 1980, Pirkle and Boeder 1977 and Pirkle and Rinaldi 1977).

1.3.4. Other Methods of Enantiomeric Analysis.

X-ray crystallography, which was first used by Bijvoet *et al.* in 1951 to determine the absolute configuration of a rubidium salt, provides the means to uniquivocally assign the absolute configuration of chiral molecules. It relied on the interpretation of an anomalous dispersion effect of the X-rays caused by heavy atoms present in a crystal lattice of the sample. Modern computerized diffractometers have negated the requirement for the addition of heavy atoms and improved the speed and accuracy of the technique. Examples of central chirality (Bijvoet *et al.* 1951), axial chirality (Akimoto *et al.* 1986) and planar chirality (Vögtle *et al.* 1983) have been studied and determined by X-ray analysis.

Infrared spectrophotometry is a principal identification test in specification and pharmacopoeial standards. The spectra of enantiomers and a racemic compound most often diverge due to the fact that the environment of one molecule in the crystal of the enantiomer differs from that of the corresponding molecule in the racemic compound

(Sørensen 1990). If the optical activity of a compound is measured and plotted as a function of the wavelength, an optical rotatory dispersion (ORD) curve is obtained. If no chromophore is present the optical rotation will continuously decrease with increasing wavelength and a plain curve is obtained. However, if a chromophore does absorb within the spectral width of the measurement a Cotton effect occurs, where the curve will show one or more peaks (+) or troughs (-). The sign of the Cotton effect can then be used to correlate a compound of unknown stereochemistry with a structurally related one of known absolute configuration (Allenmark 1991a). Circular dichromism (CD) is also a spectroscopic method, based on the principle that a chiral molecule will produce a different absorption effect on a beam of right circularly polarized light to that of a beam of left circularly polarized light. This differential absorbtion can be measured as a function of the wavelength and also produces Cotton effects. Raman optical activity spectroscopy (ROA) is a recent development which can be used to discern enantiomeric conformations of e.g. proteins in solution and enantiomeric purities of pharmaceuticals down to 0.1 percent (Hall 1995).

Differential scanning calorimetry (DSC) is a quantitative analytical technique which measures the melting processes of compounds. It can be applied to analyse the chiral purity of drug substances based on the fact that an impure preparation will show a wider melting range than that of the pure compound (Fassihi 1993). Purity determination is limited to substances containing small amounts of impurities, around 0-3 %.

Gil-Av *et al.* 1966 were among the first to use gas chromatography (GC) to achieve direct optical resolution using a stationary phase composed of lauryl esters of isoleucine and chromatographing trifluoroacetyl amino acid esters. The problem with GC in achieving chiral separations with CDs is that high temperatures tend to lower analyte retention on the column which often leads to poor enantiomeric resolution. The column should be operable at relatively low temperatures yet not exhibit excessive column bleed which destroys the column packing and adversely affects the chromatography. Many GC

phases using CDs have been introduced which help to overcome these problems. Venema *et al.* 1991 separated the enantiomers of substituted alkanes and alkanoic acids on alkylated β-CD columns operated at < 60°C, Wang *et al.* 1994 used derivatised α-CD columns to separate the enantiomers of ephedrine and its metabolites in human urine, König *et al.* 1993 resolved the atropisomers of polychlorinated biphenyls using alkyl derivatised γ-CD columns, Korth *et al.* 1991 separated the enantiomers of cyanobacterial metabolites using a β-CD column and Bicchi *et al.* 1991 used permethylated α-, β- and γ-CDs dissolved in polysiloxane polymers to resolve over 130 volatile compounds such as substituted aromatics and polar alkanes.

Supercritical fluid chromatography (SFC) is a recent technique employing a mobile phase of supercritical CO_2, often combined with a polar modifier e.g. MeOH. It is mostly operated at temperatures below that of GC operation. This generally increases the possibility of chiral separations and reduces the chances of thermal racemization or enantiomeric decomposition (Petersson *et al.* 1992). SFC has been widely used for the resolution of enantiomers e.g. Siret *et al.* 1992 separated a series of β-blockers on a 'ChyRoSine-A' stationary phase, Macaudiere *et al.* 1987 used CD based HPLC columns to separate the enantiomers of naphthyl and ortho-anisylphosphine oxides and Stauer *et al.* 1988 resolved the enantiomers of propranolol using a cyano HPLC column in subcritical fluid chromatography with chiral ion-pairing reagents in the mobile phase.

1.4. Aims and Objectives.

To develope novel analyte chiral separations using a range of analytical methodologies and CD macrocycles. To compare and contrast NMR, HPLC and FSCE for their ability to chirally resolve different groups of enantiomers and to determine if the nature of the analyte:CD interactions can be established by correlation of the results obtained from these three techniques.

CHAPTER TWO
EXPERIMENTAL

2.1. Analytes and CDs Employed.

All materials were used as received unless stated otherwise.

2.1.1. Phenethylamines.

The nine racemic phenethylamines were supplied, via the Pharmazeutisches Institut der Universität Bonn, Germany, by Albert Roussel Pharma Gmbh, Mauver Pharma Gmbh, Boehringer Ingelheim, Hoechst AG and Glaxo Gmbh.

2.1.2. Propranolol Analogues.

Racemic samples of propranolol and five of its analogues as HCl salts were kindly provided by Dr. G. Bedford of Zeneca Pharmaceuticals Ltd, Macclesfield, UK.

2.1.3. Mequitamium.

A racemic sample of Mequitamium (as an iodine salt) and individual samples of its two enantiomers, were kindly provided by Mr. Brian Meakin (School of Pharmacy, University of Bath, Bath, England) from Pharma, Milan, Italy.

2.1.4. CDs Employed.

All CDs (except for *heptakis*(2,3-di-*O*-acetyl)β-CD and peracetyl β-CD) were generously donated by Wacker Chemie Gmbh (Munich, Germany) and contained less than 10%

water w/w). The CD parental derivatives were characterised in terms of their molar substitution (MS) or degree of substitution (DS), equivalent to the average number of substituent groups per glucose unit: hydroxypropyl α-CD (HP-α-CD, MS = 0.6) methyl β-CD (Me-β-CD, DS = 1.8), hydroxyethyl β-CD (HE-β-CD, MS =0.6), hydroxypropyl β-CD (HP-β-CD, MS 0.6), hydroxypropyl γ-CD (HP-γ-CD, MS 0.6). *Heptakis*(2,3-di-*O*-acetyl)β-CD and peracetyl-β-CD were produced, characterised and donated by Henning Mallwitz, Pharmazeutisches Institut der Universität Bonn, Germany.

2.2. HPLC Studies.

2.2.1. Solvents and Buffers Employed.

HPLC grade acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), triethylamine (TEA), glacial acetic acid (HOAc) and orthophosphoric acid (H_3PO_4) were all obtained from FSA, Loughborough, England. Sodium dihydrogen orthophosphate (NaH_2PO_4), disodium hydrogen orthophosphate (Na_2HPO_4), sodium dihydrogen citrate ($HO_2CCH_2C(OH)(CO_2H)CH_2CO_2Na$), potassium dihydrogen phosphate (KH_4PO), dipotassium hydrogen phosphate (K_2HPO_4), citric acid ($HO_2CCH_2C(OH)(CO_2H)CH_2CO_2H$) and sodium hydroxide (NaOH) were all obtained from BDH, Poole, Dorset.

Water was distilled and passed through a 0.45 μm cellulose nitrate filter (Whatman, Maidstone, Kent) before use. Aqueous mobile phases were also passed through these filters, whilst mobile phases containing organic solvents were filtered through 0.45 μm nylon filters (Whatman, Maidstone, Kent) before being degassed with Helium. Polar organic mobile phases were not filtered prior to degassing. Buffer pH's were checked with a Kent EIL 7020 pH meter, calibrated at pH 4 and 9.

2.2.2. HPLC Columns Employed.

Two LC-ABZ columns (250 x 2.1 mm and 250 x 4.6 mm, 5μm) containing novel silica surfaces, which are deactivated using a combination of steric/hydrophobic groups, were kindly donated by Supelco (Bellefonte, PA, USA). A C8 Inertsil column (100 x 2.1 mm, 5μm) was obtained via GL Sciences Inc., Tokyo, Japan; a porous graphitic carbon based column, the Hypercarb S (100 x 4.6 mm), was donated by Shandon Scientific Ltd., Cheshire; a β-CD bonded column, Cyclobond I 2000 (250 x 4.6 mm, 5μm) and a γ-CD bonded column (250 x 4.6 mm, 5μm) were obtained from Technicol, Cheshire, UK; pKb pre-columns (20 x 4.6 mm, 5μm) were obtained from Supelco. All 4.6mm i.d. HPLC columns were used at a flow rate of 1 ml/min (unless stated otherwise) and all 2.1. mm i.d. columns were used at a flow rate of 0.2 ml/min (unless stated otherwise).

Inline filters with 0.5 μm frits, were used in all instances with the LC-ABZ and Inertsil columns.

2.2.3. Solute Preparation.

All solutes were dissolved using either mobile phase or acetonitrile (all analytes in the polar organic work). Where dissolution was incomplete, samples were sonicated and filtered through a 0.2 μm Acrodisc (FSA, Loughborough, UK) prior to injection.

2.2.4. HPLC Equipment.

Most work was carried out using an SSI model 300 LC pump (State College, PA, USA), a Rheodyne 7125 injection value (Cotati, CA, USA) and an SSI 500 UV detector connected to a Servogor 220 chart recorder. Other models used included a Milton Roy

3100 UV detector, a Spectromonitor III UV detector and an LDC Constametric III Pump. Injection loops of either 10μl or 20μl were employed with 10μl or 50μl syringes (SGE, Milton Keynes) for sample injections (0.05 - 0.5 mg/ml).

All HPLC columns were thermostatted at 20°C (unless stated otherwise) using a water bath containing a Grant water heater. To conduct sub-ambient temperature experiments, ice was added to the water and the solution stirred without using the heating function of the Grant water heater. Temperatures were checked using a partial immersion thermometer fitted to the side of the bath.

All samples were detected at their UV maxima (unless stated otherwise). Where no literature value was obtainable, solutions of the analytes were scanned between 190 and 360 nm using a photodiode array detector (Spectromonitor 5000, LDC Analytical).

2.2.5. Determination of Chromatographic Parameters.

1. Capacity factors, k′, were calculated in the following way;

$$k' = (t_r - t_0)/t_0 \qquad \text{(Equation 2.01.)}$$

where t_r is the analyte retention time and t_0 is the retention time for a solute unretained under the experimental conditions (generally calculated from the injection of water).

2. Selectivity, α. The selectivity between two peaks was calculated thus;

$$\alpha = k'_1 / k'_2 \qquad \text{(Equation 2.02.)}$$

where k'_2 is the capacity factor for the least retained of two enantiomers.

3. Column efficiency, $N_{w½}$, was calculated according to equation 2.03.;

$$N_{w½} = 5.54\ (t_r/_{w½})^2 \qquad \text{(Equation 2.03.)}$$

where $w_{½}$ is the peak width at half peak height

4. Chromatographic resolution, R_s, was calculated either according to equation 2.04. or equation 2.05. (for less clearly resolved peaks);

$$R_s = 2\ (t_{r2} - t_{r1}) / (w_2 + w_1) \qquad \text{(Equation 2.04.)}$$

where w_2 and w_1 are the peak widths measured at the baseline.

$$\%CRF = f/g\ X\ 100/1 \qquad \text{(Equation 2.05.)}$$

where **f** is the vertical distance from the mid-point between the two peak apices to the lowest point of the connecting peak trough and **g** is the vertical distance from the mid-point between two peak apices to the baseline. Baseline resolution of two peaks then corresponds to a %CRF of 100%.

2.3. FSCE Studies.

All the solvents and buffers employed in the FSCE studies were of the same specification and from the same sources as the HPLC work (section 2.2.1.). All samples and buffers were filtered through a 0.2 μm cellulose nitrate or nylon filter (Whatman, Maidstone, Kent) and centrifuged for 5 mins (at 10,000 rpm) before use. Columns were conitioned by flushing with distilled water, MeCN and the appropriate running buffer before analysis of the samples.

2.3.1. CE Equipment and Columns Used for Phenethylamines.

Work was carried out on a BIORAD HPE CE system, using a Biorad coated-capillary of internal diameter 25 μm and a total length of 20 cm. Samples were loaded by electromigration and separated at room temperature using a constant current of 12 μA. Data were recorded at the analyte λ_{max} value with the Biorad 800 HRLC system, version 2.30. Samples of compounds 1-9 were prepared by dissolution in acetonitrile - potassium dihydrogen phosphate (0.1M, pH 3.0) (10:90 v/v) at about 0.5 mg/ml. Buffers at pH 3 to 6 for CE were all prepared from potassium dihydrogen phosphate at 0.1M using freshly distilled and filtered water and adjusted with orthophosphoric acid or 1M sodium hydroxide. The buffer at pH 7.5 was a 50:50 mixture of 0.1M potassium dihydrogen phosphate and 0.1M dipotassium hydrogen phosphate, adjusted with 1M sodium hydroxide. See section 4.2. for experimental results and discussion.

2.3.2. CE Equipment and Columns Used for Propranolol Analogues.

A BIORAD HPE CE system was used with a BioRad coated capillary of internal diameter 25 μm and a total length of 20 cm. Samples were loaded by electromigration and separated at room temperature at a constant current of 5 μA. Data were recorded at the analyte λ_{max} values (288 nm for compounds **1-3** and 254 nm for compounds **4** and **5**) with the Biorad 800 HRLC detector, version 2.30. Samples of compounds **1-5** were prepared by dissolution in MeOH-50 mM potassium dihydrogen phosphate pH 3.0 (30:70 v/v) at about 0.5 mg/ml. Buffers were prepared from potassium dihydrogen phosphate (50 mM) using freshly distilled and filtered water and then adjusted to the appropiate pH with orthophosphoric acid before the addition of methanol. The required amount of CD was then added to the buffers. See section 4.3. for experimental results and discussion.

Electroendosmotic flow (EOF) measurements were attempted according to recommendations from the manufacturers BIORAD. A neutral marker, acetone, was loaded at the outlet reservoir in a running buffer to acetone ratio of 20:1. The polarity was set from negitive to positive and the time taken for the neutral marker to pass from the outlet reservoir to the detector window was measured (a distance of 4.6 cm). The λ_{max} was 264 nm, the maximum for acetone in an aqueous acid.

2.3.3. CE Equipment and Columns Used for Mequitamium.

Work was conducted using a Dionex CES CE system (Sunnyvale, CA, USA) and bare fused silica capillaries of internal diameter 50 μm and a total length of 50 cm. Samples were loaded by gravity (10 to 15 seconds at 90 mm) and separated at room temperature at a constant voltage of 15 KV. Data were recorded at the analyte λ_{max} (254 nm) using the inbuilt UV detector and captured using the Dionex CES software. Samples of mequitamium and its enantiomers were prepared in MeCN-water (10-90% v/v).

The electroendosmotic flow rate (u_{eo}) was calculated using a solution of MeCN injected by gravity (15 seconds at 90 mm) and a constant voltage of 15 KV (the buffer used was MeCN-sodium dihydrogen citrate (50 mM, pH 3.5) (9.5 - 90.5 v/v%). The EOF value was found to be 3.8×10^{-6} cm^2 V^{-1} s^{-1} . See section 4.4. for experimental results and discussion.

2.4. NMR Studies.

All NMR studies were carried out at the School of Pharmacy and Pharmacology, University of Bath, Bath, England, except for the Job plot with etilefrine HCl, which was

carried out in the Pharmazeutisches Institut der Universität Bonn, Bonn, Germany with the kind help of Henning Mallwitz and Ulrike Holzgrabe.

2.4.1. NMR Solvents.

Deuterium oxide (D_2O), methanol-d_4 (CD_3OD), methyl sulphoxide-d_6 (DMSO-d_6) and acetonitrile-d_3 (CD_3CN) were obtained from Aldrich (Gillingham, Dorset) or Sigma (Poole, Dorset).

2.4.2. NMR Conditions.

Cyclodextrins were dried *in vacuo* over P_4O_{10} before use and all chemical shifts were referenced to the HDO signal at 4.65 ppm. For the measurement of cyclodextrin-induced chemical shifts, coupling constants and the separation between signals arising from the individual enantiomers ($|\delta_R - \delta_S|$), sufficient quantities of all compounds with and without the appropriate cyclodextrin were dissolved in deuterated buffer to give approximately equimolar concentrations of each. Separated signals were not attributed to the respective enantiomers for the compounds. All the NMR experiments with the phenethylamines and propranolol analogues (see 5.2. and 5.3. respectively) were obtained using a Jeol EX400 FT NMR spectrometer operating at 399.05 MHz for ^1H. 64 scans with a frequency range of 5000 Hz were collected into 32K data points giving a digital resolution of 0.31Hz/pt. NMR experiments with mequitamium (see section 5.4), except for the COSY work, were obtained using a 270 MHz Jeol JNM-GX-270 instrument with 32K data points and a spectral width of 3001.2 Hz giving a digital resolution of 0.18 Hz/pt.

Where required, an appropriate Gaussian function was applied before Fourier transformation to enhance spectral resolution. The temperature for those experiments

conducted with the 400 MHz instrument was controlled at 30(±1)°C and the residual protonated water signal was suppressed using homo-gated secondary irradiation (decoupler off during data acquisition).

Analysis of AB quartet signals (in ABX systems) was performed using equation 2.01.,

$$\vartheta_0\delta = \sqrt{(f_1-f_4)(f_2-f_3)} \qquad \text{(Equation 2.01.)}$$

where f_1-f_4 are the four lines of the AB quartet. Defining Z as the centre of the multiplet (i.e. the midpoint between f_1 and f_4 or f_2 and f_3), the frequencies of each doublet of the quartet is given by,

$$\vartheta_A = Z - \vartheta_0\delta \qquad \text{(Equation 2.02.)}$$

$$\vartheta_B = Z + \vartheta_0\delta \qquad \text{(Equation 2.03.)}$$

where ϑ_A is the higher field doublet and ϑ_B is the lower field doublet.

2.4.3. Job Plots and Association Constant, K_f, Determination.

The ^{1}H NMR experiments for the Job plots and measurement of complex formation constants with etilefrine HCl (see section 5.2.) were performed on a Varian XL 300 FT NMR spectrometer operating at 299.956 MHz with a sample temperature of 26°C. Five solutions in deuterated 0.1 M phosphate buffer pH 4.5 having molar ratios (etilefrine HCl:CD) of 0.5:1, 0.75:1, 1:1, 1.25:1 and 1.5:1 with a total concentration of 14 mM were prepared for the Job plots.

The Job plots with Mequitamium (see section 5.4.) were were conducted with the 400 MHz instrument in the School of Pharmacy and Pharmacology, Bath, England. The molar ratios of analyte to CD employed (in deuteratedMeCN - sodium dihydrogen citrate buffer, 50 mM, pH 3.5) were 3/4:1/4, 2/3:1/3, 1/2:1/2, 1/3:2/3 and 1/4:3/4 with a total concentration of 12 mM. Association constants were measured using solutions with analyte:CD ratios of 1:2.33, 1:3.0, 1:4.0, and 1:5.7 for etilefrine HCl:β-CD and Ac-β-CD. Ratios of 1:1.7, 1.3.3, 1.4.7 and 1:8.3 were used for mequitamium:γ-CD and HP-β-CD.

2.4.4. Interpretation of Spectra.

Most spectra were assigned by inspection and with data from the literature. In the case of etilefrine HCl (see section 5.2.) the two aromatic doublets (H4' and H6') were distinguished using the 400 MHz instrument with the aid of inverse ^{1}H-^{13}C correlation spectroscopy with a HMQC pulse sequence on a 12 mM solution using a final data matrix of 512 x 512 real data points. Frequency widths of 5000 Hz (^{1}H) and 30120.5 Hz (^{13}C) gave row and column resolutions of 9.77 and 58.8 Hz respectively. 16 scans were accumulated per slice.

It was necessary to perform a ^{1}H-^{1}H COSY, a ^{1}H-^{13}C COSY, a broad-band decoupled ^{13}C and two DEPT (*D*istortionless *E*nhancement by *P*olarization *T*ransfer) experiments (90 and 135°) to unequivocally assign the spectra for mequitamium (see section 5.4). The ^{1}H-^{1}H COSY was obtained using the 400 MHz instrument operated at 12 KHz with a frequency range of 3 KHz collected into 1024 data points giving a digital resolution of 6.28 Hz/pt. Pulse widths were 7.8, 3.9 and 10 μs with a 1s pulse delay. 16 scans were collected at an ambient temperature of 21.2 °C. The ^{1}H-^{13}C COSY was obtained using the 400 MHz instrument operated at 8 KHz with a frequency range of 13 KHz collected into 2048 data points giving a digital resolution of 12.82 Hz/pt. Pulse widths were 10,

20 and 20 μs with a 1s pulse delay. 16 scans were collected at an ambient temperature of 20.8°C.

The broad-band decoupled ^{13}C and DEPT experiments were conducted using the 270 MHz instrument operated at 67940.6 KHz for ^{13}C with a frequency range of 18 KHz collected into 32K data points giving a digital resolution of 1.1 Hz/pt. 700 scans were collected at an ambient temperature of 20°C.

2.5. Molecular Modelling Parameters.

All molecules were constructed using the Discovery program, version 2.7.0. and Insight II program version 2.0.0. (Biosym Technologies, San Diego, CA), run on the Personal Iris from Silicon Graphics. The molecular structures of β-CD and Ac-β-CD were constructed using the building subroutine. Energy minimization of the geometries and conformations was iteratively performed until the energy deviations were less than 1 kcal/mol. See section 5.2.4. for experimental results and discussion.

CHAPTER THREE
HPLC RESULTS AND DISCUSSION

3.1. Introduction.

Optical resolution by chromatography is possible through reversible diastereomeric association between a chiral environment introduced into a column and solute enantiomers. The multiplicity of experimental conditions under which direct chromatographic optical resolutions have been achieved with CDs suggests that the difference in association which is necessary can be achieved via many types of molecular interactions as detailed in the following sections.

3.1.1. CDs as Stationary Phases in Liquid Chromatography.

Initial efforts to use CDs as stationary phases in LC were attempted by Wiedenhof *et al.* 1969, who managed to separate some benzoic acids in a non-chiral application, with polymerized β-CD gels. Harada *et al.* 1978 and Zsadon *et al.* 1979 used various crosslinked CD gels for the separation of mandelic acid derivatives and amino acids respectively. However, because of their low efficiency and poor mechanical strength, these polymer gels were unsuitable for HPLC.

Japanese researchers led the way in attempts to bond CDs to silica gel in order to overcome the problems found with the polymers. Fujimura *et al.* 1983, Kawaguchi *et al.* 1983, Tanaka *et al.* 1984 and Feitsma *et al.* 1985, used propylamine, ethylene diamine and related binding agents in their work. However, the first hydrolytically stable CD phase was introduced by Armstrong 1985, who patented the process which uses silanes (e.g. 3-glycidoxypropyl trimethoxy silane) to form a linkage between the silica and CD hydroxyls of between 2 to 20 carbon atoms in length. These packings were soon commercialized as the Cyclobond™ columns and are now available with α, β and γ-CDs.

Armstrong *et al.* 1985a separated over eighty structural, geometric and epimeric isomers of a variety of compounds (e.g. polycyclic aromatics and prostaglandins) using the same β-CD columns which they had developed. Hinze *et al.* 1985 were able to resolve the enantiomers of a series of ester derivatised amino acids and barbiturates on similar columns. Armstrong *et al.* 1985b were further able to separate metallocene enantiomers and also a diverse group of pharmaceuticals, including β-blockers, anticonvulsants and calcium channel blockers with a β-CD column (Armstrong *et al.* 1986). Li and Purdy 1991, Furuta and Nakazawa 1992, Friebe *et al.* 1992 ref, Park *et al.* 1992 and Piperaki *et al.* 1994 have all conducted experiments in enantiomeric resolution with a spectrum of analyte types on bonded β-CD columns. Agnus *et al.* 1994 developed a method for the detection of poorly UV-absorbing species on a β-CD column viz. six-fold enhancement of pregnanolone by indirect UV detection with testosterone/progesterone as markers. Not all researchers have settled for the columns developed by Armstrong and have chosen to make their own. Thuaud *et al.* 1993 examined phenylhydantoins and hydroxycoumarin derivatives on supports produced by condensation of β-CD molecules with epichlorohydrin, although the efficiency of their columns was relatively poor. Rizzi *et al.* 1994 used a β-CD column to resolve some amphetamines, with and without derivatization to diastereomers. They found that indirect separation i.e. prior derivatisation, produced the best results for these relatively small molecules.

Tanaka *et al.* 1983 used α-CD bonded phases to separate various isomers of disubstituted benzene, but the first report on the use of an α-CD bonded phase for enantiomeric separation in LC was produced by Armstrong *et al.* 1987. They were able to directly resolve the enantiomers of over twenty compounds including several amino acids, which had proven difficult to separate on β-CD phases, which they attributed to the inability of the analytes to adequately complex with the relatively larger cavity of β-CD. Issaq *et al.* 1988, Vigh *et al.* 1989a, and Florance and Konteatis 1991 have all used α-CD columns to investigate different isomeric analytes. Armstrong and Zukowski 1994 were able to resolve the enaniomers of pinene and camphene hydrocarbons on an α-CD phase,

which they attributed to 'shape selectivity' i.e. a tight steric fit of the guest into the CD cavity. γ-CD phases have been tested by Vigh *et al.* 1989a, Berthod *et al.* 1990 and Chang *et al.* 1990 for separating positional isomers, stereoisomers and peptides respectively. Abidi and Mounts 1994 separated methyl-substituted tocols on β- and γ-CD via a normal phase mechanism, whilst Loukas *et al.* 1994 determined the stability constants of a novel insecticide with a γ-CD phase. However, most reports on native CDs still use the cheap and easy to obtain β-CD.

Modified CD phases have also been investigated for LC methods. Tanaka *et al.* 1984 used acetylated versions of α- and β-CDs to separate positional isomers of aromatics, with the acetyl β-CD giving improved peak shapes over the parent macrocycle. Barnaby and MacLeod 1991 employed an acetyl-β-CD column to separate enantiomeric aza-bicycles following a novel complexation with borane. Other derivatised CD phases include hydroxypropyl-β-CD (Stalcup *et al.* 1990), *S*- and *R*-naphthylethylcarbamate-β-CD (Vandenbosch *et al.* 1992 and Berthod *et al.* 1992), and toluoyl-β-CD (Lee *et al.* 1992). Li and Purdy 1992 developed some novel derivatives of β-CD, whereby regiospecific modification was carried out by attaching groups on the narrow primary side of the CD torus, to produce 'multiple-interaction' phases.

3.1.2. Optimization of Enantioselectivity on a Bonded CD Column.

The first important consideration for analyte retention and possible chiral recognition in the reversed phase mode is proper fit of the molecule into the CD cavity. In the presence of aqueous mobile phases the basic mechanism which retains solutes is known as inclusion complexation (Technicol 1992). It is essential for the analyte to have at least one aromatic ring to facilitate the hydrophobic interactions which are believed to drive the inclusion complexation process (Allenmark 1991). If the solute is of a suitable size, contact with the internal CD surface may restrict its motion, so that differential

interaction with the mouth of the cavity will cause a difference in enantiomeric association constants, K_f, and k′ values (see equations 3.03. and 2.01. respectively).

MeCN and MeOH are the two most commonly used organic modifiers in CD bonded columns, although others have been tried (Street 1987). MeCN has a stronger solute displacement effect from the CD cavity and can generate different selectivites to that of MeOH (Li and Purdy 1992). The organic modifier competes with the analyte for the CD cavity and if it is present in too high a concentration, it will reduce both retention and chiral recognition. Anigbogu *et al.* 1992 reported the formation of ternary complexes between CDs, alcoholic modifiers and aromatic hydrocarbons during fluorescence studies, where the stoichiometry depended on the CD type (β or γ). These ternary mixtures enhanced complexation with β-CD using an achiral column in reversed phase HPLC.

pH can play an important role in chiral recognition as well as affecting analyte k′ values. On altering pH, Armstrong *et al.* 1990 found three different types of solute retention behaviour occurred with a series of tobacco alkaloids, dependant on the analytes basicity. They concluded that the protonated species were less strongly complexed by the CD support. Li and Purdy 1992 came to a similar conclusion upon examining some dansyl amino acids on derivatised CD columns, as did Ventura *et al.* 1994 using a biphenylactic acid on various CD columns. However Konishi *et al.* 1994 observed that interaction between β-CD and a carboxylic acid analyte was promoted when the analyte was present as a carboxylic ion. They related this to an improved solute ability to hydrogen bond with the hydroxyls at the primary CD end. Lee *et al.* 1992 found that pH changes caused k' values to decrease for dansyl amino acids over a pH range where the ionic form of the analyte should not have altered. They suspected changes to the stationary phase surface were responsible. Overall, the influence of pH on chiral recognition can be somewhat variable.

Under reversed-phase conditions buffers are known to include into the CD cavity and as the buffer concentration increases, solute peaks may become sharper with a concomitant reduction in k′ values. Triethylamine (TEA), Ammonium nitrate (NH_4NO_3) and citrate are recommended for use with Cyclobond™ columns (Technicol 1992). TEA has been shown to be beneficial for a number of enantiomeric separations (Lee *et al.* 1992 and Li and Purdy 1991).

β-CD columns show a greater sensitivity to temperature variations than standard reversed phase columns. Reductions in temperature can lead to an improvement in separation factors (α), but this may not necessarily improve the solute resolutions due to poor efficiency as a result of slower mass transfer. Hinze *et al.* 1985 came to such a conclusion when examining dansylamino acids on a β-CD column with varying temperatures. Aboul-enein *et al.* 1988 found that temperature had little effect on the separation factor (α) with nomifensine, although the resolution and k′ values did increase over the same decreasing temperature range (8°C to 30°C). Armstrong *et al.* 1985b and more recently Furuta and Nakazawa 1992, have shown that inclusion complexation is prevented at temperatures higher than 60°C for most analytes, a phenomenon that could prove useful when separating tightly bound solutes. Cabrera and Ludba 1994 described how changes in temperature can increase or decrease separations depending on the enthalpy and entropy contributions of the individual analytes.

3.1.3. CDs as Stationary Phases in the Polar Organic Mode.

This is a recent development in CD research and there are relatively few published papers. The presence of polar groups on the analytes may be more important for enantio-recognition in the polar organic than in conventional CD reversed phase methods. All the chiral analytes to have been separated so far contain at least two hydrogen bonding sites (donor and/or acceptor). The mobile phase consists of MeCN as the primary solvent

(90 to 99 % v/v) with MeOH present as a secondary modifier, principally to adjust retention. Acid (glacial acetic, HOAc) and base (e.g. triethylamine, TEA) are used in combination to control selectivity, with some effect on retention.

Changes in analyte retention behaviour were noticed by Chang *et al.* 1985 when examining organometallic compounds at a high MeOH content (60 to 80%) on a β-CD column and also when they tested some substituted phenols under similar conditions (Chang *et al.* 1986). They believed that the high organic modifier content was causing the column to display some form of 'normal phase' behaviour with the analytes unlikely to enter the CD cavity in such non-polar environments. Han and Armstrong 1987 discovered that upon increasing the MeCN content with a β-CD column, both retentions and chiral resolutions initially decreased but then began to increase again at very high levels (95 % MeCN). This reversal suggested a change in the separation mechanism and in chiral recognition, which they also related to some form of 'normal phase' adsorption to the external hydroxyls of the CD. Seeman *et al.* 1988 likewise observed such a reversal of retention and chiral recognition values at high MeCN concentrations (>80%). Armstrong and Jin 1989 were able to separate a series of mono-, di- and trisaccharides using β- and α-CD columns with mobile phases containing high MeCN levels (usually >80 %). These seem to be the first applications of the so called 'polar organic' methodology.

Zukowski *et al.* 1992 found they could improve on separations obtained under reversed phased conditions for FMOC derivatised imino acids, by employing a naphthylethylcarbamate-β-CD column with nonaqueous mobile phases, consisting of MeCN, HOAc and TEA. They also proposed the existance of external absorption (no cavity inclusion of the analytes) at the CD mouth and appear to have coined the term 'polar organic'. Armstrong *et al.* 1992 performed a systematic study of the effects of polar organic mobile phase composition on the retention and enantio-selectivity of a series of β-blockers. They tentatively proposed the novel idea of the analyes sitting

astride the CD torous 'like a lid' and interacting via hydrogen bonding, to account for the difference in separations observed between this mode and the normal phase. Other work by Zukowski *et al.* 1993 and Chang *et al.* 1993 have supported this idea.

3.1.4. CDs as Mobile Phase Additives in Reversed Phase Liquid Chromatography.

Upon the addition of CD to the mobile phase in HPLC, analyte molecules may undergo inclusion complex formation much as in the same way with bonded CD columns i.e. the solute enters the CD cavity via hydrophobic forces, which may lead to a stereospecific interaction with the hydroxyl groups on the mouth of the CD torous. There is often a reduction in solute k′ values when the diastereomeric complex formed is more hydrophilic than the free analyte. Differences in the adsorption of analyte:CD complexes on the stationary phase and/or differences in the complex K_f values (see p. 45 for K_f definition), is believed responsible for any observed chiral resolution (Sybilska *et al.* 1986). This may result in a reversal of elution orders when comparing a CD containing mobile phase to a CD bonded column, as observed by Casy *et al.* 1991 when studying a series of phenothiazines.

CDs were first used as eluant additives by Uekama *et al.* 1977a in gel chromatography, although not for chiral resolution. Later, Hinze and Armstrong 1980 were able to separate a series of structural isomers using CDs as additives and in 1982 Debowski *et al.* were able to separate mandelic acid enantiomers by HPLC using β-CD dissolved in the mobile phase with an achiral column. Similarly, Tanaka *et al.* 1985 employed native and methylated α- and β-CDs to separate *o*, *m* and *p* isomers of disubstituted benzenes, Mularz *et al.* 1988 used β-CD to resolve pseudoephedrine enantiomers, Shimada *et al.* 1988 separated isomeric estrogens with β-CD and Zukowski *et al.* 1988 resolved some chiral barbituates with methylated β-CDs. More recently Shimada and Hirakata 1992 separated a range of derivatised amino acids using β-CD but their results with dipetides

were not so good, and Lamparczyk *et al.* 1994 successfully resolved norgestrel enantiomers with β-CD at low temperatures (0°C). .

Various CDs have been employed as mixtures in the mobile phase. Gazdag *et al.* 1988 used α-, β- and γ-CD as additives for the adjustment of isomeric k′ values in HPLC. They suggested this was a rapid way to determine which CD would best complex with a given analyte as any CD which did not interact with a solute would not change the analyte k′ value. Sybilska *et al.* 1992 used β-CD and permethylated β-CD together to improve on enantioselectivity and shorten the k′ values of a group of therapeutic compounds. All the analytes except one (mephenytoin) showed an improvement in their separations. γ-CD (Takeuchi 1992, Dodziuk *et al.* 1994 and Roussel and Favrou 1993), α-CD (Zukowski 1991) and various CD derivatives, γ-CD and Me-γ-CD (Shimada *et al.* 1989) have also been utilised as eluent additives in HPLC.

Wännman *et al.* 1992 demonstrated the novel use of microcolumn HPLC (255 x 0.32 mm) to resolve the enantiomers of terbutaline with β-CD as an additive. They proposed that such a system would reduce material costs and enable the use of less readily available CD derivatives. Walhagen and Edholm 1991 separated enantiomers from complex biological fluids by column coupling and adding β-CD to the mobile phase with the last column. Cooper and Jefferies 1993 performed semi-preparative separation of brompheniramine enantiomers by column switching, again using β-CD as the chiral additive and obtaining optical purities >88%.

The factors affecting complexation and enantio-recognition in this mode are very similar to those operating when a bonded CD phase is used e.g. organic modifier, pH, buffer etc. One exceptional consideration is the solubility of the CD additive in the mobile phase. Taghvaei and Stewart 1991 have shown how the solubility of β-CD decreases in the presence of MeOH and DMF, whilst both DMSO and MeCN cause a solubility increase followed by an eventual decrease. MeCN at 20% v/v in aqueous solution

allowed the dissolution of 3.1 g/100 ml β-CD, more than twice its solubility in pure water. Pharr *et al.* 1989 demonstrated the ability of concentrated urea solutions to enhance the solubility of β-CD eleven-fold, while a 0.75 M NaOH solution gave a twenty one fold enhancement. It is thus possible to increase the absolute amount of CD but the high levels of these other additives may interfere with the complexation process.

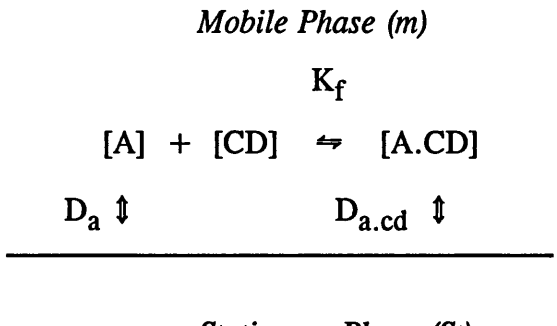
3.1.5. Equilibria in CD Modified HPLC.

The equilibria in mobile phase additive systems is somewhat more complicated than for CD bonded columns. The solute may be complexed by more than one CD molecule, with differentiation of the eluting transient diastereomers arising as a result of :

- differences in association (stability) constants (K_f) of CD:analyte complexes
- and/or differences in adsorption of CD:analyte complexes on to the stationary phase

These factors may act in an additive or subtractive manner. Uekama *et al.* 1978a and 1978b proposed the following equilibria (Fig. 3.1.) to describe the important effects occurring when using CDs as eluant additives in reversed phase LC.

Figure 3.1. Equilibria model for effects of CDs in reversed phase LC.



Distribution ratios D_a, $D_{a.cd}$ and the complex formation constant, K_f are given as follows,

- $D_a = [A]_{st}/[A]_m$ (Equation 3.01.)
- $D_{a.cd} = [A.CD]_{st}/[A.CD]_m$ (Equation 3.02.)
- $K_f = [A.CD]_m/[A]_m.[CD]_m$ (Equation 3.03.)

where [A], [CD] and [A.CD] are the equilibrium concentrations of solute, cyclodextrin and complex respectively, with $_{st}$ and $_m$ denoting the stationary and mobile phases. The observed distribution ratio, D_{obs}, is given by;

- $D_{obs} = (D_a - D_{a.cd} \cdot K_f \cdot [CD]_m)/(1 + K_f.[CD]_m)$ (Equation 3.04.)

Assuming that the addition of CD does not change the stationary phase, the distribution ratios can be related to chromatographic k′ values viz.

- $D_a = k'_a \cdot V_m/V_{st} = (t_a - t_0/t_0).(V_m/V_{st})$ (Equation 3.05.)
- $D_{a.cd} = k'_{a.cd} \cdot V_m/V_{st} = (t_{a.cd} - t_0/t_0).(V_m/V_{st})$ (Equation 3.06.)
- $D_{obs} = k'_{obs} \cdot V_m/V_{st} = (t_{obs} - t_0/t_0).(V_m/V_{st})$ (Equation 3.07.)

where V_m/V_{st} is the phase volume ratio, t_0, t_a, $t_{a.cd}$ and t_{obs} are the retention times of an unretained solute, uncomplexed solute (no CD present), complexed solute and observed solute at equilibrium, respectively. Substitution of equation 3.04. and rearrangement then gives,

- $[CD]_m/(t_a - t_{obs}) = (1/t_a - t_{a.cd}).[CD]_m + (1/K_f.(t_a - t_{a.cd})$ (Equation 3.08.)

Plotting $[CD]_m/(t_a-t_{obs})$ vs $[CD]_m$ gives a straight line with slope equal to $1/(t_a-t_{a.cd})$ and intercept of $1/K_f.(t_a-t_{a.cd})$, which allow the k′ values of the complex and the association constant to be obtained. Sybilska *et al.* 1986 derived an analogous equation,

- $k' = (k'_A - k')/[CD].K_A + k'_{A.CD}$ (Equation 3.09.)

where a plot of k′ vs $(k'_A - k')/[CD]$ readily allows the determination of K_A, the stability constant (1/slope) and $k'_{A.CD}$, the complex k′ values (intercept). Inherent in these mathematical treatments are the suppositions that:

1. only one species of the solute takes part in adsorption and complexation
2. complexes of 1:1 stoichiometery are formed
3. the CD does not influence the nature of the stationary phase i.e. the adsorption of CD itself onto the stationary phase is very small or negligible.

However these conditions may not always hold true and other treatments have been developed to account for this.

Sybilska *et al.* 1982 have produced an expression for weak acids and bases;

- $$k' = \frac{k'_G + k'_{iG}.K_a/[H+] + k'_{G.cd}.K_G[CD] + k'_{iG.CD}.K_{iG}[CD]K_a/[H+]}{1 + K_a/[H+] + K_G[CD] + K_{iG}.[CD]K_a/[H+]}$$

(Equation 3.10.)

where K_a is the acidity constant, K_G and K_{iG} are stability constants of CD complexes with neutral and ionic species respectively and k'_G, k'_{iG}, $k'_{G.cd}$ and $k'_{iG.cd}$ are capacity factors of unionized, ionized, complexed-unionized and complexed-ionized solute forms respectively.

The mobile phase frequently contains an organic modifier, whose competitive influence on the complexation equilibria has been described by Zukowski *et al.* 1985;

- $[CD]_m = [CD]m^o/K_{solv}[solv]m^o + 1$ (Equation 3.11.)

where $[solv]m^o$ is the initial molar concentration of organic solvent and K_{solv} is the stability constant of the 1:1 CD inclusion complex with an organic solvent. The apparent CD molar concentration, $[CD]_m$, is therefore smaller than the overall molar concentration, $[CD]m^o$. This obviously affects any values calculated from equation 3.08.

3.1.6. CDs as Mobile Phase Additives with Porous Graphitic Carbon Columns.

Porous graphitic carbon (PGC) was first produced by Kaur and Knox in 1984 and its structure elucidated by X-ray diffraction and other related means two years later (Kaur 1986 and Knox *et al.* 1986). It was made by impregnating porous silica with phenol and hexamine, heated to form a carbonized resin, which was then heated further to 2500°C after the silica had been dissolved away. The resulting material has a two dimensional structure of closely intertwined layers imparting the mechanical stability and rigidity necessary for use in HPLC. It possesses an extremely uniform surface devoid of any 'functional sites' and can be used over the entire pH range (Lim 1992). It is more hydrophobic than ODS phases and consequently larger amounts of organic modifier are required for solute elution (Kaur 1990). The retention mechanism is strongly influenced by the unique electronic donor-acceptor interactions between analytes (donor) and the delocalized electron conduction bands (acceptor) of the PGC surface (Bell *et al.* 1994).

PGC is not optically active but it is sensitive to stereochemical changes in solutes due to its flat surface leading to the preferential retention of planar molecules (Tanaka *et al.* 1993). Fell *et al.* 1988 were the first to demonstrate the ability of a β-CD containing

mobile phase to resolve the enantiomers of lorazepam and oxazepam on a PGC column (they also found that such a degree of separation could not be achieved using a bonded β-CD column, Clark 1989). Mama *et al.* 1989a were able to separate nomifensine enantiomers at pH 2.5 using β-CD and Clark and Mama 1989 used a pH of 2.5 to optically resolve glycyl-DL-phenyl alanine. These last two applications demonstrate how the unique ability of PGC to operate at extremes of pH, can prove highly beneficial.

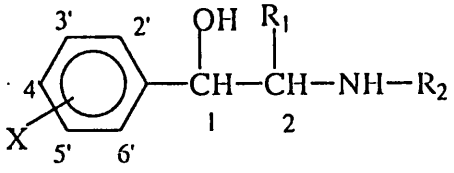
The paucity of enantiomeric separations on PGC using CD additives may be related to the rather poor solubility of α-, β- and γ-CD in eluents containing high levels of organic modifier. However Clark and Mama 1989 pointed the way in the use of CD derivatives, when they separated the enantiomers of chlorpheniramine by adding Me-β-CD to the mobile phase. Many CD derivatives are more soluble than the parent macrocycles, making them amenable for dissolution in the high organic content PGC mobile phases.

3.2. Phenethylamines - Results and Discussion

A series of nine structurally related chiral sympathomimetic agents (Fig. 3.2.) were chosen for examination with a variety of CD derivatives. These adrenergic ligands may display different isomeric potency ratios at β and α-receptors (Casy, 1993a). *l*-Noradrenaline, for instance, acts as an α-adrenergic full agonist, whilst the inactive *d*-isomer acts as a competitive α-adrenergic antagonist. Furthermore, *rac*-isoprenaline and *rac*-salbutamol are known to exhibit clear differences in intrinsic activity towards β-receptors (Krstulovic 1989a).

Consequently, great interest lies in developing means to separate the enantiomers of these compounds and they can also function as probes for the investigation of complex formation mechanisms with CDs.

Fig. 3.2. Structure of Phenethylamines.



Compound	name	X	R_1	R_2
1	cphedrinc	H	CH_3	CH_3
2	oxedrine	4-OH	H	CH_3
3	oxilofrine (oxyephedrinc)	4-OH	CH_3	CH_3
4	norfenefrine	3-OH	H	H
5	etilefrine	3-OH	H	CH_2CH_3
6	orciprenaline	3,5-diOH	H	$CH(CH_3)_2$
7	noradrenaline (norepinephrine)	3,4-diOH	H	H
8	isoprenaline	3,4-diOH	H	$CH(CH_3)_2$
9	salbutamol	3-CH_2OH,4-OH	H	$C(CH_3)_3$

3.2.1. β-CD Column in Reversed Phase Mode.

Several HPLC methods for the determination of enantiomeric purity of certain phenethylamines have been developed; chiral stationary phases (Wainer *et al.* 1983, Schill *et al.* 1986 and Wu *et al.* 1990), a chiral complexing agent as liquid stationary phase (Pettersson and Stuurman 1984) or pre-column derivatization to form diastereoisomers (Gal 1984 and Noggle and Clark 1986). However, there seem to be no reports of chiral separation using CD columns without prior derivatization. It was only possible to resolve the enantiomers of orciprenaline, ephedrine and oxilofrine (Table 3.1.), which also displayed the three longest k′ values.

Table 3.1. Resolution values and k′ values of phenethylamines with a β-CD column. HPLC conditions: 0.5% MeCN- 99.5% v/v TEA (0.8 % v/v, pH 8.0), β-CD bonded column (250 x 4.6 mm), 1 ml/min, Temp. 20°C. See equation 2.05 in section 2.2.5. for %CRF calculation procedure.

Compound	k′ values	% CRF value
Ephedrine	4.3, 4.4	36%
Oxilofrine	3.5, 3.8	67%
Oxedrine	2.8	0
Norfenefrine	2.1	0
Etilefrine	2.9	0
Isoprenaline	2.9	0
Noradrenaline	2.3	0
Orciprenaline	3.2, 3.4	39%
Salbutamol	2.2	0

Ephedrine and oxilofrine are the only compounds with a methyl group on the C2 carbon which seems then to be influencing their separation. Orciprenaline does not have a methyl on this carbon, but does possess hydroxy groups in a unique 3',5' substitution pattern on the aromatic ring. Hydrophobic / steric and hydrogen bonding phenonema, optimum for interaction with the tethered β-CD molecules, may account for the optical resolution and longer k′ values of these three compounds.

3.2.2. Polar Organic Investigations.

The phenethylamines examined here possess one or more hydrogen bonding and/or acceptor sites (similar to the propranolol analogues in section 3.3.) and so may be

expected to display some enantio-discrimination when using this mode of separation in conjunction with a β-CD bonded column (Technicol 1992).

Despite employing a range of varied experimental conditions, only the enantiomers of salbutamol were successfully separated to any degree (Table 3.2.), which strongly suggests that the mode of interaction between analyte and CD is significantly different in the polar organic mode to that in the conventional reversed phase situation, which gave contrasting results (section 3.2.1.). Salbutamol is the most bulky and hydrophobic of the phenethylamines and such structural features were also found to promote enantio-recognition for the propranolol analogues in the polar organic mode (section 3.3.2).

Table 3.2. Resolution and k′ values for phenethylamines in polar organic mode. HPLC conditions: mobile phase, MeCN-MeOH (97.5-2.5 % v/v), HOAc-TEA (0.3-0.2 %v/v), β-CD column (250 x 4.6 mm), 1 ml/min, Temp. 20°C.

Compound	k′ values	% CRF value
Ephedrine	43	0
Oxilofrine	46	0
Oxedrine	> 100	-
Norfenefrine	> 100	-
Etilefrine	52	0
Isoprenaline	80	0
Noradrenaline	>100	-
Orciprenaline	90	0
Salbutamol	59, 62	32

3.2.3. CDs as Mobile Phase Additives - Reversed Phase Mode.

Mularz *et al.* 1988, were able to resolve the enantiomers of pseudoephedrine but not ephedrine when using β-CD dissolved in the mobile phase in conjunction with an achiral

cyano-bonded analytical column. They attributed this to the differing ability of the analytes hydroxyl and ammonium groups to hydrogen bond to the 2' and 3' hydroxyls of the β-CD molecule. There are no reports in the literature of further enantiomeric separations of phenethylamines using the above methodology.

Perhaps not surprisingly then, no resolution was found for any of the phenethylamines using a range of CDs with an achiral column viz. β-,α-, Me-β, HE-β, HP-β, HP-α-CD or peracetyl β-CD. β-CD produced the largest drop in k′ values of the analytes when dissolved in the mobile phase while α-CD produced the smallest reduction. These results may suggest that chiral discrimination interactions between the analyte and CD are not occurring here but the use of β-CD in capillary electrophoresis experiments (section 4.2.) and peracetyl β-CD in PGC work (section 3.2.4.) has proven that such interactions could be happening in solution here and that their detection is linked to the technique in use.

3.2.4. Porous Graphitic Carbon (PGC) Column.

Clark and Mama 1989 have used modified CDs as additives to the mobile phase for the enantiomeric separation of analytes on a PGC phase. Here a series of mobile phases containing different CDs was examined for the ability to provide enantio-separation for the phenethylamine compounds on PGC, which has been shown to exhibit marked differences in retention characteristics for a series of phenol derivatives compared to a standard ODS column (Forgács *et al.* 1993). For these reasons it may be expected to facilitate unique chiral separations commensurate with its novel surface structure.

Only orciprenaline, ephedrine and oxilofrine could be enantiomerically separated when using a β-CD containing mobile phase. The use of HE-β-CD, Me-β-CD, HP-β-CD and α-CD produced no resolution for any of the analytes. Peracetyl β-CD, previously unreported in use with a PGC column, gave a significant improvement on the results

obtained with β-CD although it did not generate additional chiral separations (Table 3.3).

Table 3.3. Results obtained with β-CD and peracetyl β-CD containing mobile phases on a PGC column. HPLC conditons: (A) MeCN - Na_2HPO_4 (3 mM), (30-70 %v/v), pH 3.0 containing 2.5 mM β-CD *or* (B) MeCN - Na_2HPO_4 (3 mM), (36-64 %v/v), pH 3.0 containing 2.5 mM peracetyl β-CD. PGC column (100 x 4.6 mm), 0.8 ml/min, Temp.20°C.

	HPLC conditions (A)		HPLC conditions (B)	
Compound	k′ values	%CRF value	k′ values	%CRF value
orciprenaline	1.8, 2.3	20	1.3, 2.3	78
ephedrine	3.8, 4.0	< 5	4.0, 4.5	45
oxilofrine	2.8, 3.3	35	3.3, 3.8	82

Peracetyl β-CD is unable to donate a proton when hydrogen bonding and it is insoluble in water, making it amenable for use with a PGC column, where mobile phases need to contain high levels of organic modifier to reducing analyte k′ values which then also facilitates the dissolution of peracetyl β-CD. This hydrophobic environment could promote otherwise weak hydrophilic forces and help explain the improved chiral recognition of peracetyl β-CD over the other, more hydrophilic CDs examined.

3.2.5. Comparison of HPLC Results for Phenethylamines.

The peracetyl β-CD mobile phase in conjunction with a PGC column provided the most effective overall means of HPLC separation for the phenethylamines, with very short k′ values and relatively high resolution values obtained for ephedrine, oxilofrine and orciprenaline but not for the other analogues (see Table 3.3.).

Although the β-CD bonded column also resolved these three analytes (Table 3.1.), the k′ values were longer than the PGC column and the resolution values were lower. All the analytes clearly displayed some affinity for the unmodified β-CD molecule (as assessed from their retention data), whether it was bound to the column surface (section 3.2.1.) or added to the mobile phase used with a PGC column (section 3.2.4.). However the solute resolution values found with the β-CD column clearly show that its chiral discrimination ability was the lesser of these two.

The presence of peracetyl β-CD as an additive with a standard ODS column did not result in any enantio-recognition, in contrast to the results on a PGC column. This can be attributed to the lack of retention of the analytes and/or the transient nature of the diastereomeric complexes, whose interaction with the ODS surface was reduced by having >35% of organic modifier in the mobile phase, which was necessary to dissolve the water-insoluble peracetyl β-CD to any significant extent. In fact no resolution was observed for any of the analytes using an ODS column with CDs as mobile phase additives (section 3.2.3.). Clearly the selectivity of the ODS and PGC stationary phases must be different as neither β-CD nor peracetyl β-CD produced any resolution with the same analytes. The unique two-dimensional surface of PGC, consisting of closely intertwined ribbons of graphite, is highly sensitive to steric changes that disturb the electron density of solute molecules (Lim 1992). Consequently its influence on the separation of optical isomers will be greater than that of the ODS phase where solute hydrophobicity is the dominating factor in the partition mechanism (Tanaka *et al.* 1993).

Only the salbutamol enantiomers could be separated in the polar organic experiments (section 3.2.2.). The absence of any correlation between the k′ values and selectivity results obtained in this mode with those of the other three methods, indicates that the polar organic chromatographic mechanisms are considerably different to those which operate in less hydrophobic environments. Recent publications have indeed suggested this to be the case (Zukowski *et al.* 1992 and Chang *et al.* 1993).

3.3. Propranolol Anologues - Results and Discussion.

Propranolol [1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol] is a widely prescribed optically active β-blocker used in the treatment of various cardiovascular disorders. The R and S enantiomers of propranolol are known to exhibit different pharmacological effects *in vivo* (Krstulovic 1989b) , which has led to considerable interest in developing chromatographic methods to separate its enantiomers (Haginaka *et al.* 1992).

This section presents the range of capacity factors, k′, and enantio-selectivities observed for a series of structurally related compounds based on the drug propranolol (**1**; see Fig. 3.3.), when interacting with a range of CD molecules. Of the six compounds investigated, four were naphthyl derivatives (**1**, **2**, **3** and **6**) and two were phenyl derivatives (compounds **4** and **5**). Each of these compounds had either an *i*-propyl or *t*-butyl group on the secondary amine, except for compound **6**, which possessed a unique CONH-ethyl group and compound **3**, R = ethyl. These analogues were manufactured by Zeneca Pharmaceuticals with the aim of identifying additional β-blocking drugs. It is thus of importance to identify chromatographic means of separating their enantiomers due to the strong possibility of the chiral forms displaying differing effects *in vivo*.

Figure 3.3. Structures of compounds 1-6.

$$R' - O - CH_2 - CHOH - CH_2 - NH - R$$

Compound **1**, R′ = naphthyl, R = *i*-propyl Compound **4**, R′ = phenyl, R = *i*-propyl
Compound **2**, R′ = naphthyl, R = *t*-butyl Compound **5**, R′ = phenyl, R = *t*-butyl
Compound **3**, R′ = naphthyl, R = ethyl
Compound **6**, R′ = naphthyl, R = CONH-ethyl

3.3.1. β-CD Column in Reversed Phase Mode.

Propranolol itself has been chirally resolved by HPLC using various bonded stationary phases, such as cellulose tris-3,5-dimethylphenylcarbamate (Vandenbosch *et al.* 1992), an ovomucoid bonded phase (Haginaka *et al.* 1992) and a cellulase silica phase (Marle *et al.* 1991).

It has however proved to be much more difficult to separate propranolol using bonded CD columns in the reverse phase mode. Thuaud *et al.* 1993, did manage to separate its enantiomers using a novel epichlorohydrin-β-CD-ammonium modified macrocycle, with a 20% MeOH-phosphate buffer at pH 4, although the resolution was low (R_s = 0.5) and the column efficiency was poor. Armstrong *et al.* 1986 improved the chiral resolution of propranolol using two β-CD bonded phase columns joined in series with a mobile phase of 25% MeOH-Triethylammonium acetate (TEAA) (1% v/v), pH 4.1. Due to the prohibitive cost of obtaining two β-CD columns, work in this laboratory used one such column with a view to examining how the structural differences of compounds **1-6** would affect their possible chiral resolution within a range of mobile phases and operating temperatures.

No resolution could be observed here for propranolol using the mobile phase listed by Armstrong *et al.* 1986. It was expected that at least some resolution would occur, albeit with only one β-CD column in use, but the fact that none did so suggests that there were substantial selectivity differences between the columns employed in both cases. Only compound **2** showed any resolution (11.5 %CRF) with one β-CD column and the mobile phase conditions used by Armstrong *et al.* 1986. The MeOH content was lowered to increase the analyte retention times, which resulted in longer k' values as expected and caused all but compounds **3** and **6** to display some enantio-recognition (Table 3.4.).

Compound **6** (R = $CONHC_2H_5$) had the longest k′ values of the entire group (the retention is believed to be due solely to interaction with the bonded β-CD molecules), yet it showed no chiral resolution, whilst **2**, the most non-polar of the analytes, eluted in half the time and displayed a clear %CRF of 34%. Obviously the structure of the analyte's R group (both with R′ = naphthyl) is important for their chiral recognition, which occurred in the order **2** > **1** > **3/6** (**3** and **6** %CRF= 0 (see Fig. 3.4. on p. 58).

Table 3.4. k′ values and %CRF values (in parenthesis) of compounds 1-6. HPLC conditions: β-CD column (250 x 4.6 mm), flow rate 1 ml/min. Mobile phase: *X* % MeOH-TEAA (1% v/v) pH 4.1. Temperature 25°C.

X % MeOH	1	2	3	4	5	6
25% MeOH	0.6 (0)	0.55, 0.48 (11.5)	0.57 (0)	0.3 (0)	0.3 (0)	1.2 (0)
5% MeOH	1.8,1.9 (5)	1.9, 2.0 (34)	1.7 (0)	0.66 (*)	0.67, 0.73 (30)	4.5 (0)

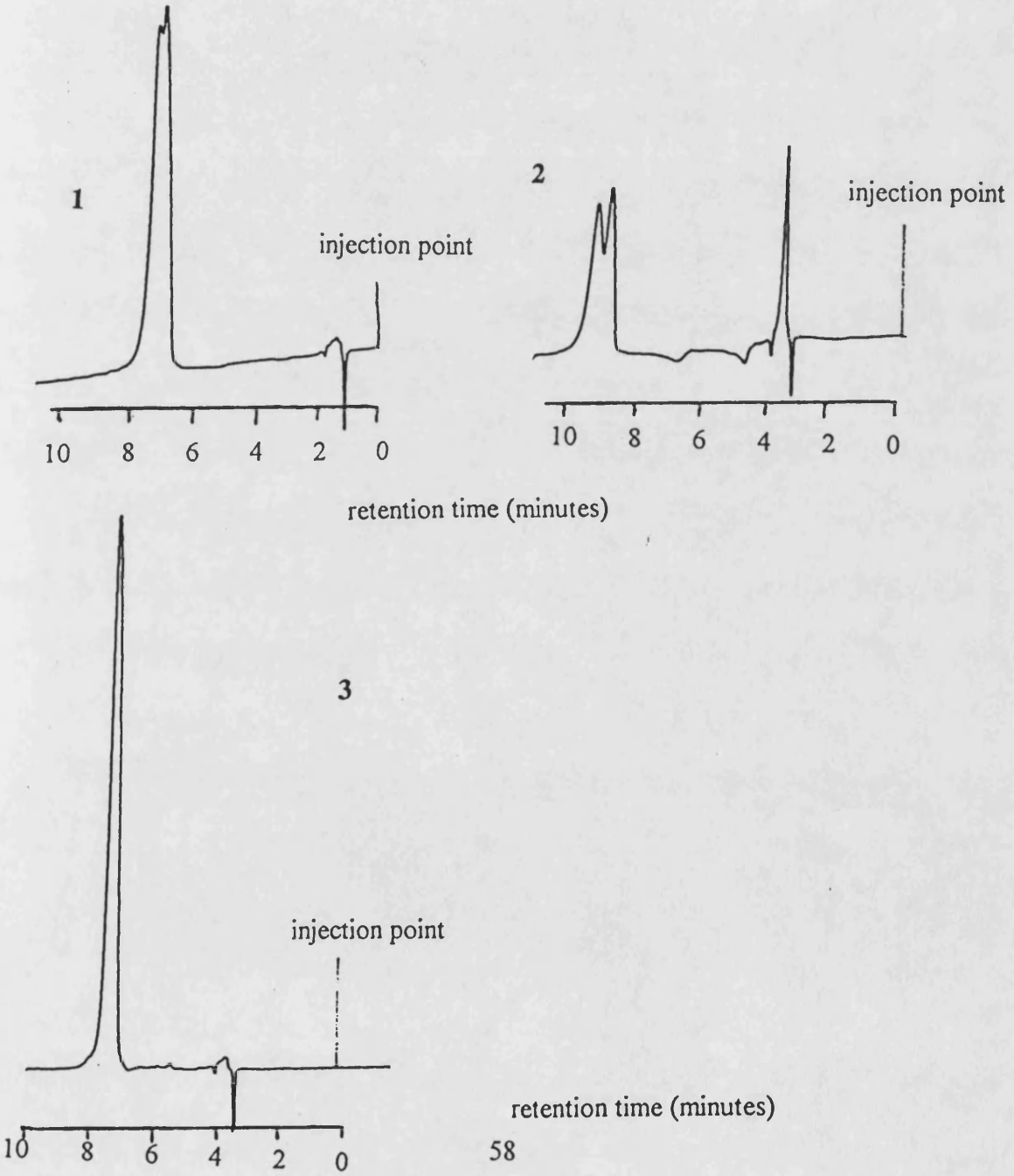
* peak shoulder too small to measure %CRF.

Lowering the %MeOH further, changing the percentage of TEAA or altering the pH did not result in any significant improvement to the %CRF values of the compounds. It was then decided to vary the temperature and observe its effect.

Lowering the temperature can cause an improvement in chiral resolution when using CDs (Aboul-Enein *et al.* 1988) by increasing the k′ values of the enantiomers due to an enhancement of the interaction between the CD stationary phase and the eluting analyte molecules i.e. an increase in the CD:analyte binding constant (Ahuja 1991). This can in turn cause an improvement in the chiral recognition process. In view of this effect, the

temperature of the chromatographic system was varied between 10°C and 35°C and the results on k′ values and %CRF are shown in Figs. 3.5 and 3.6., respectively on p. 59.

Fig. 3.4. Chromatograms of compounds 1, 2 and 3 showing various degrees of chiral separation. HPLC conditions: β-CD column (250 x 4.6 mm), flow 1 ml/min. Mobile phase: 5% MeOH-Triethylammonium acetate (1% v/v) pH 4.1, Temp. 25°C.



All the compounds showed a decrease in k′ values as the temperature was raised from 10°C to 35°C. Compounds **1**, **2**, **4** and **5** demonstrated a reduction in %CRF values over the same increasing temperature range (Fig. 3.6.). Compounds **3** and **6** showed no enantio-separation under any of the conditions used here.

Fig. 3.5. and 3.6. k′ values and %CRF values for 1-6 with changing temperature. HPLC conditions as Fig. 3.4.

Fig. 3.5.

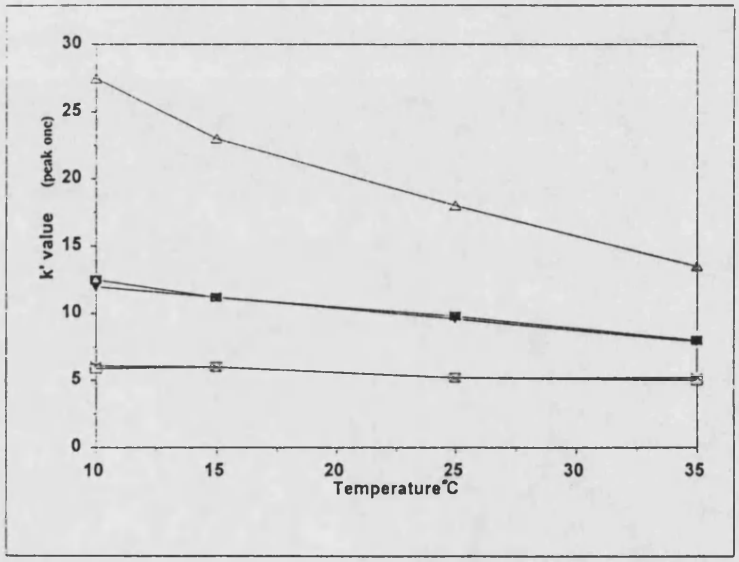
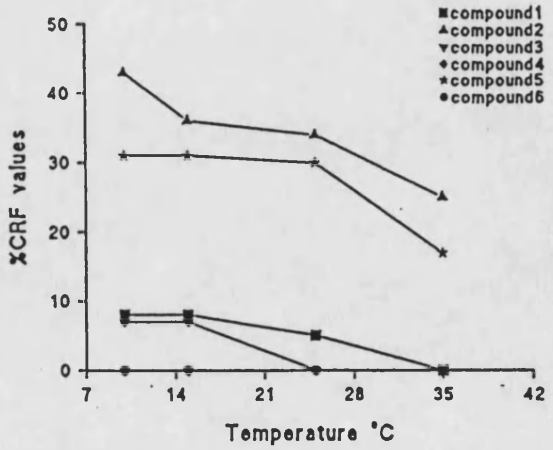


Fig. 3.6.



Only compounds **5** and **2** still showed chiral resolution at 35°C. It was also seen that decreasing the temperature from 15°C to 10°C resulted in no further improvements in resolution for any of the analytes, except in the case of compound **2**.

Three bands occurred in the van't Hoff plots (log k′ vs 1/T °K) for the compounds (see Fig. 3.7. on p.61). The fact that some of the compounds bunched together in this manner suggests a similarity in retention mechanisms, engendered by their relative structural similarities viz. compounds **1**, **2** and **3** (naphthyl group with R = *i*-propyl, *t*-butyl and ethyl respectively) produced one set of van't Hoff plots very close together as did compounds **4** and **5** (phenyl group with R = *i*-propyl and *t*-butyl), whereas compound **6** (naphthyl group with a urea linkage) produced a distinctly separate plot from these others. It was further noted that the plots were relatively non-linear ($r < 0.9900$), except for those of compounds **6** and **3**, which showed straighter lines ($r = 0.9972$ and 0.9925 respectively).

According to Horvath *et al.* 1976, non-linear plots can be expected whenever any one of the following three conditions holds;

(i) the eluate exists in two or more forms having a different retention,
(ii) there exists two or more retention mechanisms as a result of the heterogeneity of the stationary phase surface containing more than one type of binding site,
(iii) both (i) and (ii) are true.

It would appear here that condition (iii) is likely to be true as each analyte has two different 'forms' (R and S configuration, which can interact differently in a chiral environment even though they may not be chromatographically resolved) and the β-CD stationary phase is known to operate using a variety of binding forces, such as hydrophobic interactions, van der Waals forces and hydrogen bonding (Piperaki *et al.*

1994). The fact that compounds **6** and **3** (with R and S configurations) have more linear van't Hoff plots than the others (coupled with their lack of optical resolution) suggests that they may be primarily interacting with the β-CD molecules via only one main driving force e.g. hydrophobicity. The long alkyl chain of **6** may be unfavourable for a chiral discriminatory interaction with the mouth of the β-CD molecules as the nitrogen nuclei of the urea group are less amenable to hydrogen-bond formation than the amine functions of the other analogues. Thus the 'three point interaction' model proposed by Dagleish 1952 would be more unlikely to occur, explaining its lack of optical resolution.

According to Issaq *et al.* 1987 when the percentage decrease in retention times (with a β-CD column) are the same for two or more analytes upon increasing the temperature, the interactions of the analytes with the stationary phase can be assumed to be similar. Table 3.5. shows how the % decrease in retention times for compounds **1** to **3**, and **4/5** are very close.

Fig. 3.7. van't Hoff plots for compounds 1-6. HPLC conditions as in Fig. 3.6.

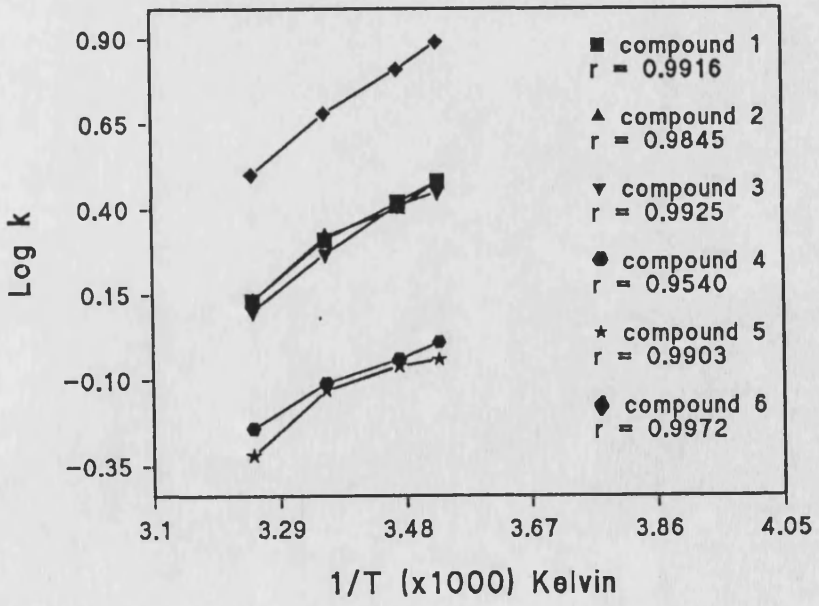


Table 3.5. The % decrease in retention times (mins) for compounds 1-6 upon increasing the temperature from 10°C to 35°C. HPLC conditions as in Fig. 3.6.

Compound	1	2	3	4	5	6
% decrease in retention time (mins)	58.9%	58.4%	59.3%	78%	77.7%	47%

The value of the % decrease in retention time for compound **6** is much lower (only 47%) than that of the other analytes, which demonstrates, in conjunction with the van't Hoff plot (Fig. 3.6.), that its interaction with the β-CD stationary phase is significantly different to the other compounds.

Those analytes with a bulky non-polar *t*-butyl group (i.e. compounds **2** and **5**) displayed the largest %CRF, with compound **2** always having the higher value (see Fig. 3.6.). The larger naphthyl moiety of compound **2** may allow for a tighter fit of the analyte inside the CD cavity (explaining its consistently longer retention than that of compound **5**) and possibly provide for a more favourable chiral interaction of the analyte alkyl chain with the secondary hydroxyl groups of the host macrocycle.

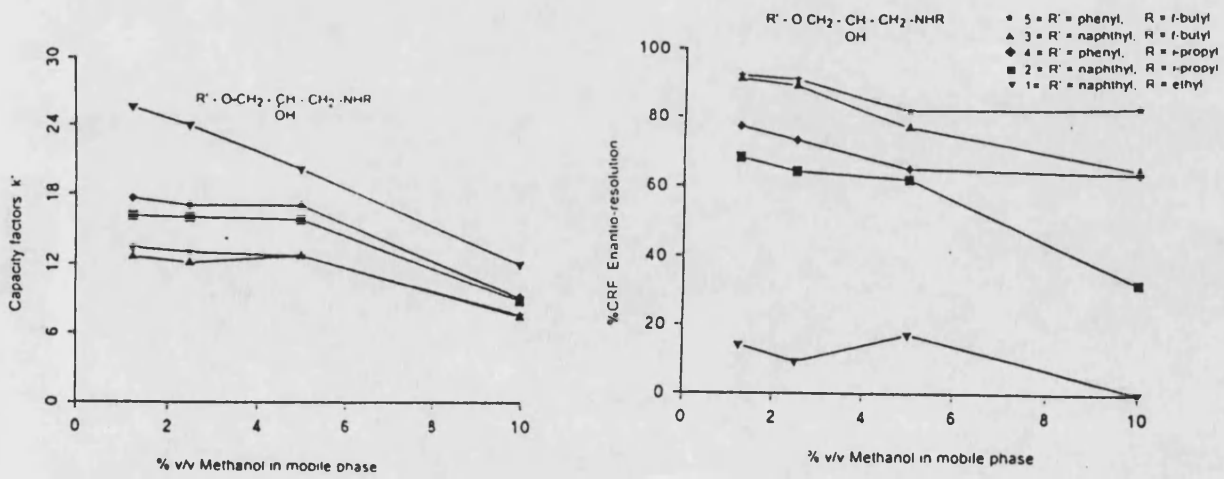
3.3.2. Polar Organic Investigations.

Initial experiments used conditions developed by Armstrong *et al.* 1992. In that work propranolol (**1**) was stated to be baseline resolved using a polar organic mobile phase of MeCN-MeOH (95-5 %v/v), containing HOAc-TEA (0.3-0.2 %v/v) with a β-CD bonded column. However such a degree of separation for compound **1**, under the same operating conditions, could not be achieved in this laboratory. Here the %CRF value for propranolol was only 22.5% as compared to the baseline (i.e. 100 %CRF) separation claimed by Armstrong *et al.* 1992.This may have been due to column efficiency differences caused by batch to batch reproducibility changes in the manufacturing process

or possible alterations to the stationary phase surface caused by extensive use of this column for previous reversed phase applications. The separation values reported here are still reasonably good however and their value lies in comparing them with the varied structures of the analytes and in suggesting the important role which hydrophobic / steric forces may still possess in relatively non-polar environments.

Increasing the amount of MeOH in the mobile phase is known to decrease the retention time of β-blocker type molecules, by virtue of competition with the analytes for hydrogen-bonding sites on the CD rim. Thus the analyte:CD interaction is reduced, Chang *et al.* 1993. This behaviour is apparent from consideration of Fig. 3.8. which shows that the k′ values of compounds **1-5** decreased with an increasing MeOH content and a concurrent fall in the MeCN concentration. A comparison of Fig. 3.8. and Fig. 3.9., in which %CRF is plotted against %MeOH, shows that all the individual analytes except **3**, displayed their highest observed %CRF in connection with their highest k′ values i.e. the stronger any one specific analyte interacted with the β-CD stationary phase, then the higher was its %CRF.

Fig. 3.8. k′ value *vs* % MeOH and Fig. 3.9. %CRF *vs* %MeOH.



It is of interest to compare the rank order of %CRF and k′ values for the group of compounds studied. When R′ was either naphthyl or phenyl, the k′ of the compounds decreased (**3** < **2** <**1** and **4** < **5**) as the size of the R group increased (ethyl < *i*-propyl < *t*-butyl). Conversely, for each subset of analogues, the %CRF showed an opppsite relationship and increased with the size of the R group (**3** > **2** > **1** and **5** > **4**, see Fig. 3.9.). It appears that stronger interactions with β-CD, as indicated by longer k′ values, do not necessarily confer enantioselectivity. Thus an increase in hydrophobicity of the R group decreases the affinity of the ligand for the macrocycle while at the same time affecting those interactions which confer enantio-discrimination. It has been noted previously (Branch *et al.* 1994) that a derivatised β-CD, which showed greater chiral discrimination than β-CD, displayed weaker binding to some phenethylamine analytes.

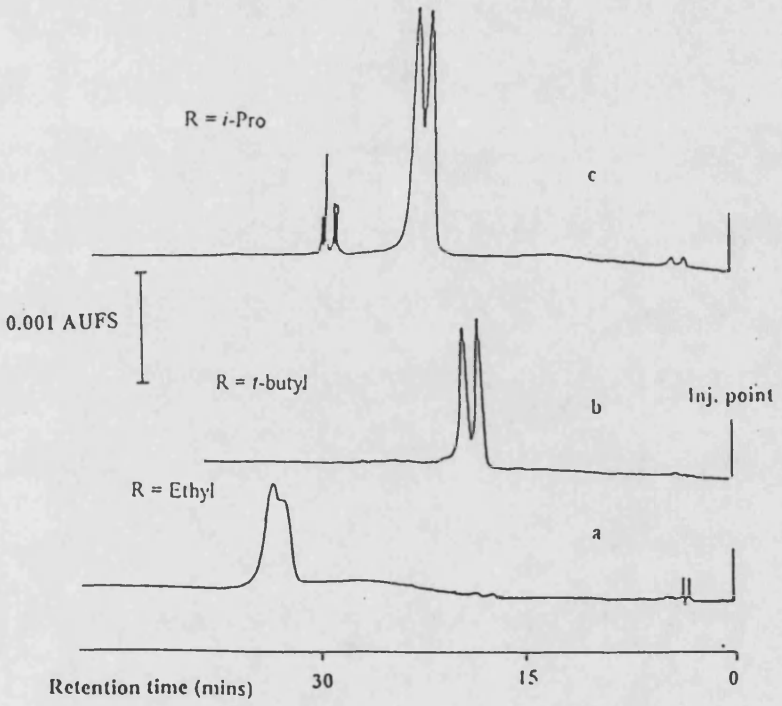
It is considered that analytes do not enter the CD cavity in the polar organic mode but rather are believed to sit over the CD like a 'lid' and interact through some process involving hydrogen-bonding (Armstrong *et al.* 1992 and Chang *et al.* 1993). As compounds **1**, **2** and **3** differ only in their R group, which is itself incapable of hydrogen-bonding, any differences seen in %CRF probably arise as a result of varied steric/hydrophobic interactions with the CD. Upon comparison of the analyte pairs **1/4** and **2/5**, it is apparent (see Fig. 3.9.) that for the same R groups, when R′ = naphthyl (as in **1** and **2**), the %CRF is lower than if R′ = Phe (**4** and **5**). Figure 3.10. on p. 64 shows the effect on resolution of a variation in the R group of compounds **1**, **2** and **3** where R′ = naphthyl and also compounds **4** and **5** where R′ = phenyl.

Fig. 3.11. on p. 66 shows the structure of the alkyl chain and the hydrogen-bonding (dashed lines) proposed to occur with the CD rim, as stated by Armstrong *et al.* 1986. If the van der Waals radius of the R group is large enough to cause steric repulsion with the CD it could explain the experimental observation that within each analyte subset,

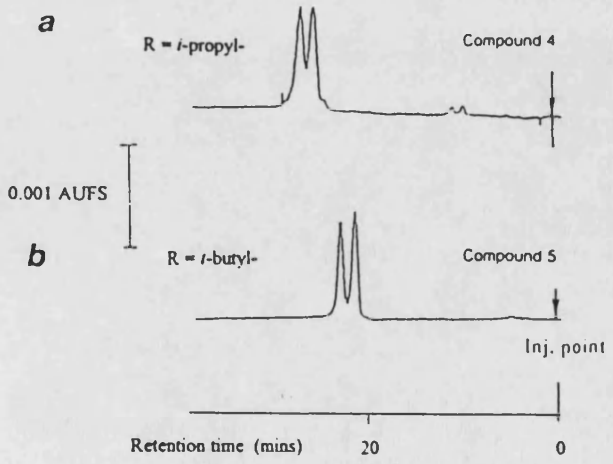
%CRF increases as R hydrophobicity (and steric bulk) increases and that k′ values simultaneously fall due to a greater repulsive effect.

Fig. 3.10. Effect of R group on retention and %CRF values. [1] (R′= naphthyl) a) **3**, R= ethyl, b) **2**, R= *t*-butyl, c) **1**, R= *i*-propyl and **[2]** (R′= phenyl) a) **4**, R= *i*-propyl and b) **5**, R= *t*-butyl. HPLC conditions: β-CD column (250 x 4.6 mm), mobile phase; MeCN-MeOH (95-5 %v/v), HOAc-TEA (0.3-0.2 %v/v) at 1 ml/min. Temp. 25°C.

[1]

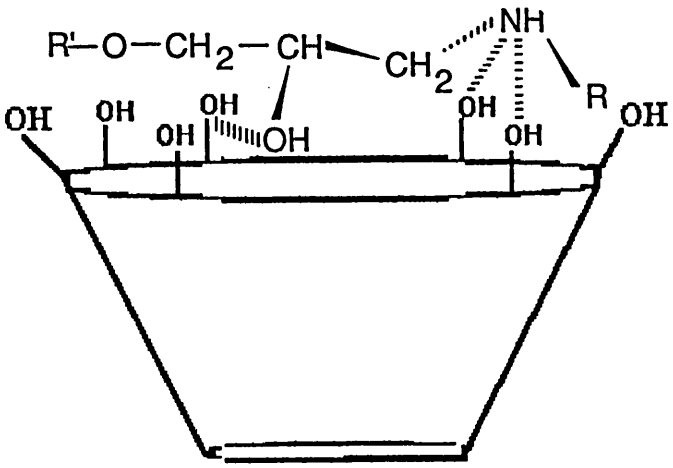


[2]



It was not possible to observe enantio-discrimination for compound **6** under any of the experimental conditions tested. This analyte, which has an extended R group containing a urea linkage, was virtually unretained in all the experiments (< 5 mins). The decreased ability of the urea group to hydrogen bond (due to the participation of its nitrogen atom's lone pairs in the resonance hybrid structure) may have been responsible for its rapid elution compared to compounds **1-5**, each of which possessed a secondary amine group.

Figure 3.11. Complexation Structure between Propranolol Analogues and β-CD.
This figure is schematic only and does not represent the actual interaction model.



Using a mobile phase of MeCN-MeOH (95-5 %v/v) the TEA and HOAc ratio was varied and the resulting effects on analyte k′ values are shown in Figs. 3.12 and 3.13. Increasing the %TEA relative to a fixed HOAc of 0.3%, caused a steady decrease in k′ values for compounds **1** to **5** (Fig. 3.12), whereas increasing the %HOAc concentration (relative to a fixed TEA of 0.2%) caused firstly an initial decrease in k′ values, followed by a subsequent increase and then finally little or no change in retention at a HOAc:TEA ratio of 4:1 (Fig. 3.13). All five analytes behaved in a similar manner. Due to the very

low retention of compound **6** it was not possible to observe any significant change in its behaviour upon altering the HOAc:TEA ratio and consequently the data is not reported here.

Figs. 3.12. Capacity factors (k′) *vs.* % TEA and 3.13. Capacity factors (k′) *vs.* % HOAc. HPLC conditions as in Fig. 3.10. with varying % of TEA or HOAc.

Fig. 3.12.

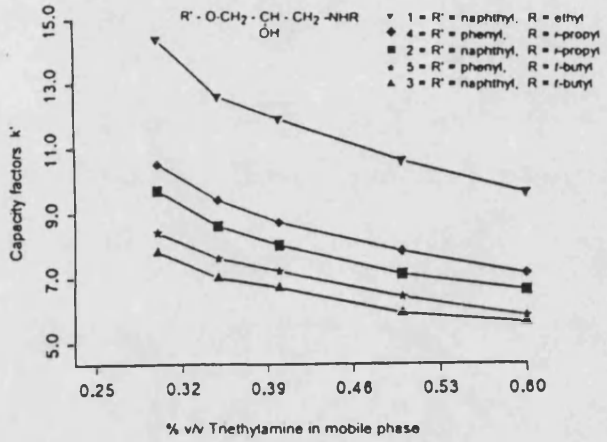
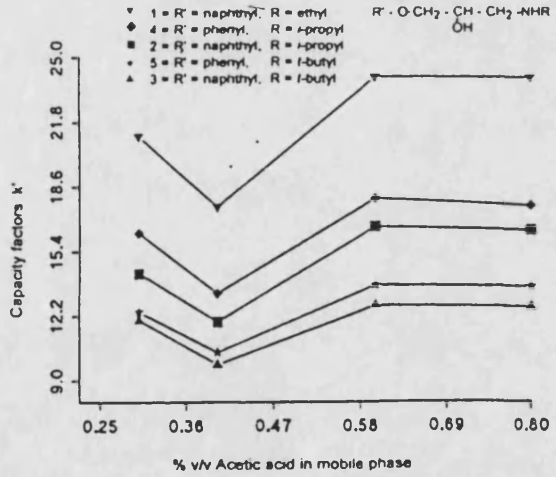


Fig. 3.13.



Clearly the ratio of HOAc:TEA controls the degree of ionization of the analytes (all of which are secondary amines) and therefore their ability to hydrogen-bond to the CD hydroxyl groups. Any reduction in the hydrogen-bonding is likely to reduce analyte retention and it would appear that increasing the HOAc:TEA ratio from 1:1 to 1:2 (Fig.

3.12) did indeed reduce the analyte-CD complexation. The TEA molecules, which are strong hydrogen acceptors, will compete with the analytes for the hydroxyl groups on the cyclodextrin rim. An increase in the TEA content of the mobile phase will enhance this competitive effect, which may explain the lower analyte k′ values. The effect on %CRF was rather muted however, with all the analytes showing little change in %CRF (see Fig. 3.14. on p.69). Factors other than hydrogen bonding would seem to also be important for enantio-recognition as implied from the rather small %CRF differences seen with these analytes when changes are made to the acid:base ratio as described above.

The initial decrease in retention time seen with increasing the HOAc to 0.4% may also have been caused by the ability of the HOAc molecules to hydrogen-bond to the CDs, but the subsequent formation of acetate salts of the analytes at relatively high acid concentrations (which are less soluble in organic solvents, Armstrong *et al.* 1992), may have accounted for the later increase in analyte retention (Fig. 3.13.). The effect on %CRF of increasing the %HOAc concentration was less straightforward (see Fig. 3.15 on p.69). Compounds **2**, **3** and **5** showed only relatively slight differences in %CRF (<12%) whereas compounds **1** and **4** (both with R = *i*-propyl), had a larger range in %CRF of 17% and 24% respectively.

Thus %HOAc had a stronger effect on %CRF than %TEA over the range studied. All the analytes, except compound **6**, gave a maximum %CRF at a HOAc:TEA ratio of 0.3:0.2 % v/v. This emphasises the proposed importance of hydrogen-bonding in the polar organic mode, as all these analytes would exist in the same ionization state at this acid-base ratio (due to their similar amine sites), which has proven to be optimum for their enantio-resolution. In reversed phase systems, alcohols are known to have a stronger association with cyclodextrins in the order PrOH > EtOH > MeOH (Technicol 1992). When MeOH was replaced by EtOH in the polar organic mobile phase, all the compounds showed a decrease in their k′ values presumably caused by a stronger

Figs. 3.14. % CRF vs %TEA and Fig. 3.15. % CRF vs % HOAc. HPLC conditions as in Fig. 3.10. with varying % of TEA or HOAc.

Fig. 3.14.

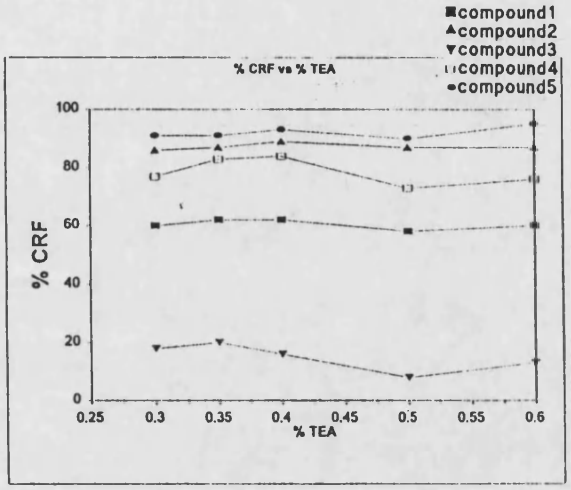
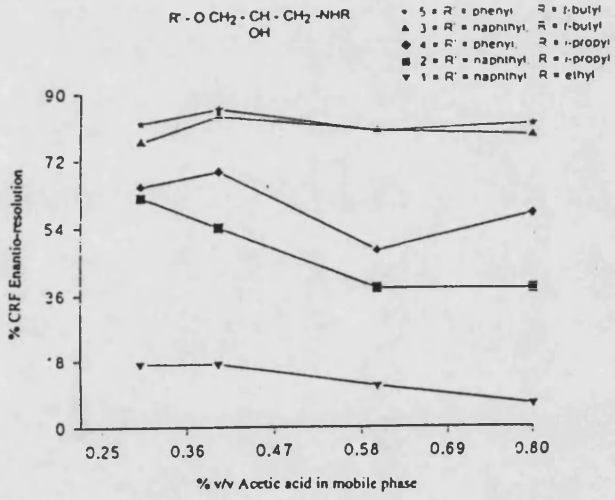


Fig. 3.15.



displacement from around the CD by the EtOH molecules (Table 3.9., see p.69). Another explanation for this decrease in k′ values may be associated with the moisture contents of MeCN (0.02%), MeOH (0.002%) and absolute ethanol (0.2%) used in these studies. Whilst the presence of 5% MeOH would not significantly change the moisture

content of the MeCN, its replacement by 5% EtOH would raise the moisture content of the mobile phase from 0.002% to 0.03%. Although it has been shown that moisture levels below 4% can dramatically reduce k′ and α values, it is not clear wether this small increase is sufficient to reduce k′ values here.

Two of the analytes (compounds **1** and **3**) showed a reduction in enantio-selectivity whilst three of them (compounds **2**, **4** and **5**) actually gave improved %CRF values. The structure of the alcoholic modifier clearly has an important influence on the overall enantio-recognition process probably through alterations to the solvation shell around the analyte and cyclodextrin, which is also likely to vary from solute to solute.

Table 3.9. Effect of MeOH and EtOH alcoholic modifiers on k′ values and enantio-resolution (%CRF) of compounds 1-6. HPLC conditions: β-cyclodextrin bonded column (250 x 4.6mm), mobile phase; MeCN-MeOH or EtOH (95-5 %v/v), HOAc-TEA (0.3-0.2 %v/v) at 1 ml/min. Temp 25°C.

	k′ values (first peak)		%CRF	
Compound	5% MeOH	5% EtOH	5% MeOH	5% EtOH
1	15.1	10.1	62	58
2	11.9	7.9	77	87
3	21.1	16.5	17	5
4	16.4	10.6	65	75
5	12.6	8.6	82	89
6	2.4	1.9	0	0

3.3.3. CDs as Mobile Phase Additives - Reversed Phase Mode.

Propranolol has reportedly been separated by Mularz *et al.* 1988, using β-CD as a mobile phase additive in RP-HPLC, although the α value was low (1.04). There appear

to be no other published reports concerning the resolution of propranolol enantiomers by this means. In this study a series of CD derivatives were dissolved in a mobile phase of MeCN-KH_2PO_4 (15-85 %v/v), 5 mM, pH 3.5 at a concentration of 12 mmol with an achiral C8 Inertsil column (10 x 0.21 cm). These conditions were chosen to allow for reasonable k′ values i.e. 5 to 20 and to minimize the amount of mobile phase needed as some of the CDs were in short supply. The Inertsil column is strongly base deactivated which makes it ideal for the study of basic compounds by preventing excessive peak tailing. Despite these measures and subsequent alterations to the chromatographic conditions (changes in the organic modifier content and pH), no chiral resolution was observed for propranolol (**1**) in the presence of any of the CD molecules. Only compound **2** displayed enantio-recognition, with a %CRF = 30 % upon addition of 12 mM HE-β-CD.

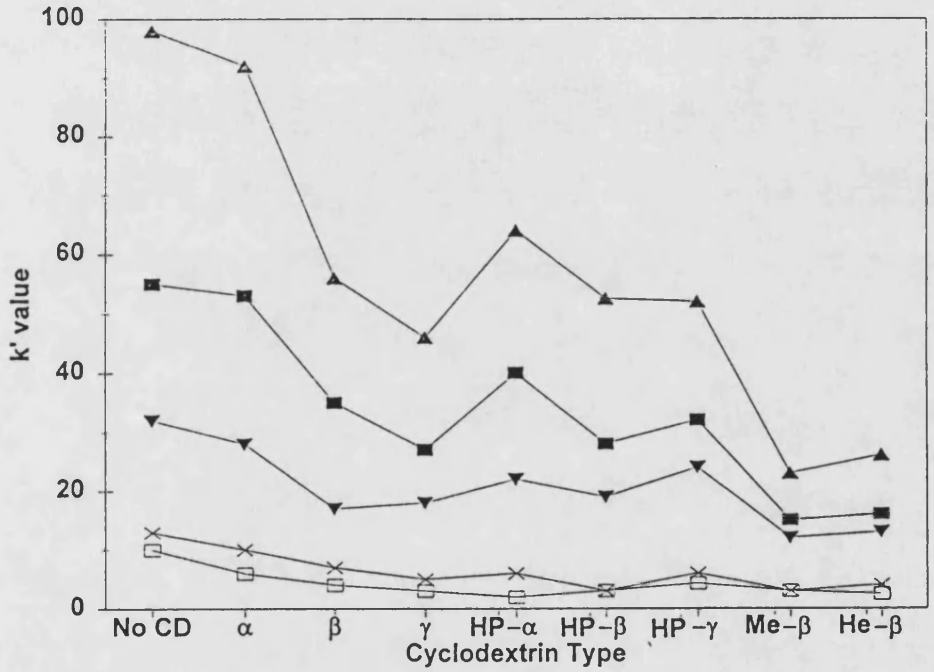
Figure 3.16. shows the k′ values of compounds **1-5** in the presence of various CD derivatives. Inclusion of the analyte by the CD molecule will tend to result in shorter retention times due to a reduction in the free analyte - stationary phase interaction. The k′ values of compound **6** were always greater than 45, giving a small and very broad peak. This suggests that this analyte interacts very little, if at all, with the CD molecules under these conditions.

Although practically no enantiospecific discrimination occurred for any of the compounds it can be seen from Fig. 3.16., on p.72, that non-specific inclusion did occur in most cases i.e. significant changes in the analyte retention were found upon addition of the various CD molecules. α-CD produced little change in the retention of compounds **1-3** which would be expected due to the large size of the naphthyl group of these analytes preventing inclusion with the smaller CD torous.

The He and Me derivatives of β-CD produced the largest reduction in k′ values for compounds **1-3**. Derivatization of the three parent CDs did therefore effect all the

analytes behaviour but no chiral resolution was observed (except in the one case noted before). Apparent increases in the analyte-CD binding (as denoted by reduced k′ values) were found to be no guarantee of optical separation.

Figure 3.16. k′ values *vs* CD type for compounds 1-5. Chromatographic conditions as stated in text.



3.3.4. Porous Graphitic Carbon (PGC) Column.

Josefsson *et al.* 1993 have resolved diastereomeric derivatives of propranolol {α(-) menthyl chloroformate} using a Hypercarb S column and Clark and Mama 1989, have stated that the enantiomers of propranolol (**1**) can be separated using a porous graphitic

carbon (PGC) stationary phase (100 x 4.6 mm) and a mobile phase of MeCN-Na_2HPO_4 3 mM (90:10), pH 3, containing 2.5 mM β-CD. Other β-blocking agents have reportedly been separated under similar conditions eg Mama *et al.* 1989 have described the partial separation of the enantiomers of metoprolol. It was thus decided to examine the propranolol analogues (compounds **1-6**) using this methodology to try to effect some novel separations with a PGC column (100 x 4.6mm, 7μm dp).

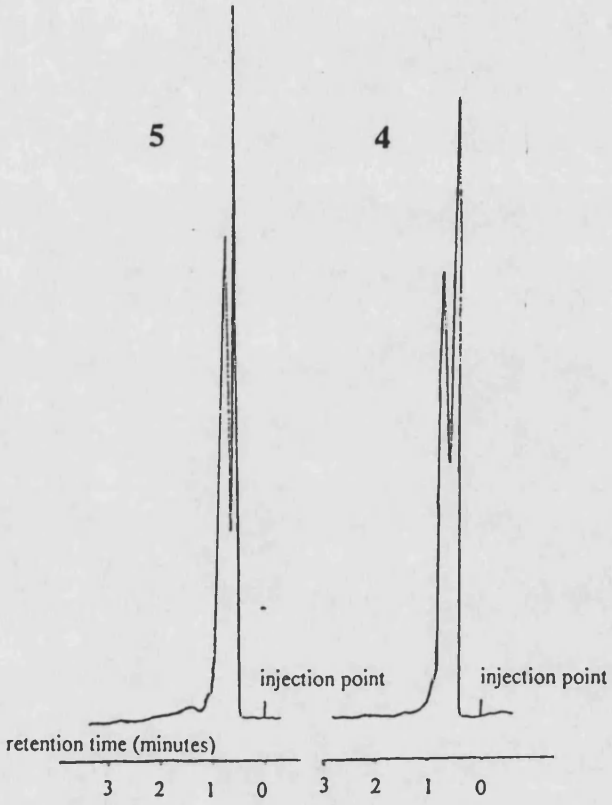
Despite repeated attempts it proved impossible to dissolve 2.5 mM of β-CD in a mobile phase with 90% MeCN so that it was not possible to reproduce the results stated by Clark *et al.* 1989 for the separation of the propranolol (**1**) enantiomers. The organic modifier content was lowered to 50% which allowed a dissolution of 5.3 mM β-CD (the maximum possible at that MeCN level according to Taghvaei *et al.* 1991) but no chiral resolution was observed for any of the compounds. Subsequent changes to the buffer concentration and pH were to no avail. The additions of HP-β-CD, HE-β-CD, Me-β-CD or β-CD, failed to produce any observable enantio-recognition for compounds **1-6**. However, the CDs did cause the k′ values of the analytes to decrease when compared to a mobile phase lacking any CD additive, indicating that some CD:analyte interaction probably did occur.

Upon addition of 2.5 mM peracetyl β-CD to a mobile phase of MeCN-Na_2HPO_4, 3 mM, (36:64 %v/v) pH 3, enantio-resolution was observed for compounds **4** and **5** (Fig. 3.17.). It would appear that the bulky naphthyl moiety of compounds **1** and **2** may be responsible for their lack of resolution as they only differ from analytes **4** and **5** in this respect. Compounds **3** and **6**, which also possessed a naphthyl group, did not exhibit any chiral resolution either.

Peracetyl β-CD is an extremely hydrophobic CD derivative (it is water insoluble), which unlike the other CDs tried, can only function as a hydrogen bond acceptor in the 2, 3 and

6 positions. Due to its hydrophobic nature high levels of organic modifier are required to dissolve it. A saturated peracetyl β-CD mobile phase of 50% MeOH-H_2O has been

Figure 3.17. Chiral resolution of compounds 4 and 5 using a porous graphic carbon stationary phase. Mobile phase: MeCN-Na_2HPO_4, 3 mM, (36:64 %v/v) pH 3, 2.5 mM peracetyl β-CD. PGC column (100 x 4.6 mm), flow rate 1 ml/min, Temp. 25°C.



shown to resolve the enantiomers of methylphenobarbital and two derivatives using a LiChrosorb RP 18 column (250 x 1 mm) (Krustulovic 1989c) with retention times of between 25 and 110 mins, whilst the slight enantio-resolution observed by Sybilska *et al.* 1988 for the enantiomers of norgestrel under similar conditions took over 35 mins. However the k′ values here were under 1.1 for compounds **4** and **5** (retention times < 1.0 mins). These very quick elution times are due to the absence of functional sites on the carbon stationary phase surface retarding the movement of the analytes (Kaur, 1991). A

porous graphitic carbon column seems then to offer an attractive alternative to the use of conventional reverse columns when high amounts of organic modifier are necessary to allow dissolution of hydrophobic mobile phase additives e.g. peracetyl β-CD.

3.3.5. Comparison of HPLC Results for Propranolol Analogues.

Of the four different chromatographic approaches used (reversed phase CD bonded, reversed phase CD additive, PGC CD additive and polar organic mode), the propranolol analogues were best separated using the polar organic methodology, employing the same β-CD column as in the reversed phase mode. β-CD as a mobile phase additive, produced no chiral resolution for any of the analytes when used with either an ODS or a PGC column. With β-CD as the chiral selector in each of the four HPLC techniques employed, the order of analyte retention (**6>2>1>3>5>4**) was identical in three modes i.e. reversed phase CD bonded, reversed phase CD additive and PGC CD additive. With the polar organic approach however, the order of analyte retention was different (**3>4>1>5>2>6**). Furthermore, the analyte resolution order on the reversed phase CD bonded column (section 3.3.3.) was different in every case i.e. in the reversed phase CD bonded mode the enantio-resolution order was compound **2 > 5, 1> 4** and **3 = 6**, but in the polar organic mode this order changed to **5 > 2, 4 > 1** and **3 > 6**.

These results suggest that the separation and retention mechanisms operating in the polar organic conditions are significantly different to those in the reversed phase situation. This is consistent with the findings of Zukowski *et al.* 1993 and Armstrong *et al.* 1992, who suggested that polar organic enantioselectivity is caused by hydrogen bonding at the CD mouth, where the analyte is positioned like 'a lid'. Hydrogen-bonding alone will not be enough for chiral discrimination in the polar organic mode. If it were, then separations would be repeatable under normal phase conditions. This is not the case (Chang *et al.* 1993). With no inclusion complexation occuring, the analyte aromatic rings will be

readily solvated in the hydrophobic solvent, whilst the alkyl chain containing polar sites and a terminal hydrophobic group, will be brought into closer contact with the cyclodextrin rim. If this terminal hydrophobic group exerts a size dependant *repulsive* steric effect with the polar cyclodextrin rim, it could, in conjunction with the hydrogen-bonding, provide the necessary 'three point interaction' required for chiral discrimination. Interactions necessary for optical resolution may be attractive or repulsive (Krstulovic 1989b).

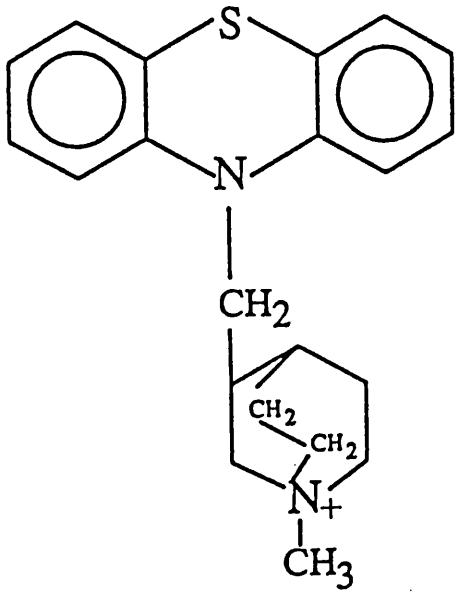
Peracetyl β-CD displayed a unique selectivity towards compounds **4** and **5** on the PGC column, but subsequent experiments on an ODS column failed to reproduce this behaviour (no analyte retention was observed by virtue of the high level of organic modifier required for dissolution of the CD). The very short retentions and yet high resolutions seen on PGC with peracetyl β-CD, demonstrate that judicial selection of the stationary phase can be as important as the choice of chiral selectand.

Only compound **2** was resolved under reversed phase conditions on an ODS column, when HE-β-CD was added to the mobile phase (section 3.3.1.). However, by virtue of the observed reductions in analyte k′ values (see Fig. 3.16.) in the presence of various CDs, it was clear that inclusion complexation was occurring in many cases. α-CD, HP-α-CD, HP-γ-CD and HP-β-CD tended to produce the smallest changes in compound k′ values. This may be linked to the 'tightness' of analyte fit within the CD cavity, where α-CD and HP-α-CD were too small to allow inclusion, while HP-γ-CD and HP-β-CD provided too large a cavity to provide for effective steric interaction. This was clearly the least successful method of chiral resolution for the propranolol analogues and explains the lack of publications dealing with this particular approach.

3.4. Mequitamium - Results and Discussion.

Mequitamium (see Fig. 3.18.) is a chiral anti-microbial compound developed for disinfecting solutions contact lenses thus aiding in the prevention of eye infections caused by the presence of certain pathogenic bacteria. Pharmacological tests have shown that the R-isomer is the most active form of the compound and it was thus desirable for the manufacturers to develop chromatographic methods to separate the enantiomers.

Fig. 3.18. Structure of Mequitamium.



3.4.1. CD Column in Reversed Phase Mode.

It was not possible to enantiomerically resolve mequitamium using a γ-CD bonded column (250 x 4.6 mm) in the reversed phase mode. Method development i.e. changing

the organic modifier concentration / type, pH, buffer and flow rates were to no avail. The analyte eluted with a k′ value < 4 with no observable chiral discrimination. The analyte was clearly interacting weakly with the stationary phase surface under most of these experimental conditions.

The chiral selector, γ-CD, may be influenced in some way when bound to the column surface in the manufacturing process. The CD is joined to the silica gel via one or more silane linkages, mostly at the narrow primary end of the CD molecule (Armstrong 1985). This connecting spacer arm will clearly restrict the orientation and motion of the γ-CD molecule, which may explain its lack of interaction with the analyte. Some workers have observed discrepancies when comparing data between CDs in the eluent and bonded phase modes (Cooper and Jefferies 1991, Armstrong *et al.* 1985).

Similar experiments with mequitamium using a β-CD bonded column also failed to produce any enantioselectivity. A comparison of the k′ values of the analyte with each bonded column type, under the same operating conditions, showed that there was no significant difference in the degree of retention.

3.4.2. Polar Organic Investigations.

In order to accomplish separations in the polar organic mode the analyte must possess more than one hydrogen donor and/or acceptor group (Technicol 1992). Examination of the structure of mequitamium (Fig. 3.18.) shows that it does not fit the criteria for enantiomeric resolution in this mode. A few initial experiments with a γ-CD bonded column demonstrated that the analyte was poorly retained using a polar organic mobile phase and that no chiral resolution occurred, as may have been expected.

3.4.3. CDs as Mobile Phase Additives - Reversed Phase Mode.

Mequitamium is a quaternary ammonium compound and will be strongly retained on any material possessing negatively charged groups, such as unreacted silanols present on a column surface. During initial achiral experiments using a highly base deactivated column (LC-ABZ, 250 x 4.6 mm) it proved impossible to elute the analyte without having at least 40% MeCN in the mobile phase. This amount of organic modifier then severely limits the amount of CD which can be dissolved in the mobile phase. In a novel solution to these problems, experiments were conducted using two pKb pre-columns (20 x 4.6 mm) joined in series as the analytical columns (Eto and Noda 1992 have used 25 or 50 mm ODS cartridges in chiral separations with β-CD mobile phases).

Using this chromatographic system the elution time of mequitamium was still over 80 minutes with a mobile phase containing 12% MeCN. Addition of various CD molecules generally produced a reduction in the analyte retention time and caused enantio-separations in two instances: with γ-CD (Fig. 3.19., see p.81) and HP-β-CD (Table 3.10., see p.80).

The addition of α-CD produced no resolution or reduction in k′ values as might have been expected, due to its rather small cavity diameter (4.7 - 5.2 Å) being unable to accommodate any portion of the much larger mequitamium structure. Two CDs, β and HP-γ, caused the analyte to elute faster than HP-β-CD and γ-CD respectively (indicating stronger complexation), yet unlike these aforementioned CDs, produced no observable enantio-resolution. Of the other three CDs tested, no chiral resolution occurred though analyte k′ values were decreased. CD inclusion around the aromatic portion of mequitamium would probably reduce analyte retention on the column, so accounting for the faster elution, but such interaction may be at a point too far removed from the analyte's chiral centre on the bulky aliphatic ring, to provide for stereospecific resolution.

When MeOH was substituted for MeCN under the mobile phase conditions stated in Fig. 3.19. (see p.81), mequitamium chiral resolution was completely removed and the k′ values increased from around 20 to 45. Selectivity differences between organic modifiers may be attributed to their solvation strengths and varied associations with the CD molecules viz. MeOH is known to be able to hydrogen-bond with the external hydroxyl groups of CDs so that it may lessen analyte interaction (Technicol 1992), which may account for the removal of mequitamium enantio-resolution and increase in k′ factor seen here.

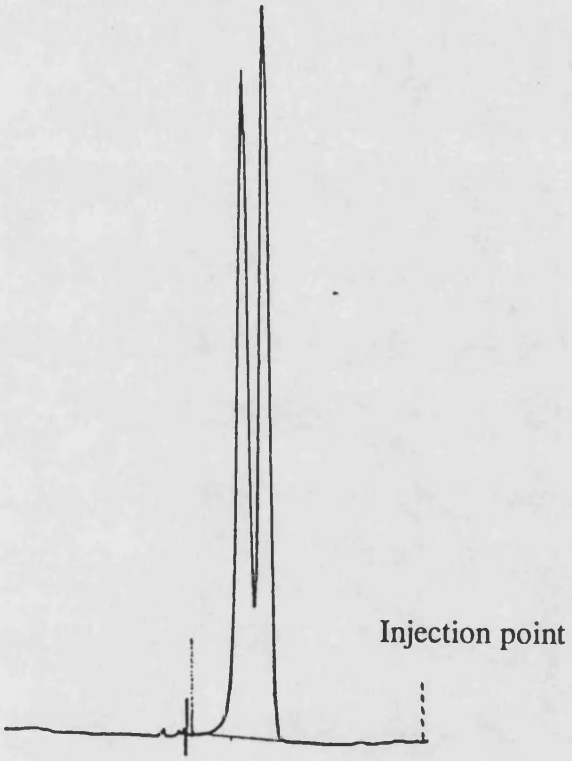
Table 3.10. k′ values and enantio-resolution values (Rs) for mequitamium. HPLC conditions: pKb pre-column (20 x 4.6 mm) x2, mobile phase 12% MeCN:88 % $NaH_2Citrate$ (10 mM) containing 12 mM of CD, 0.8 ml/min flow rate, Temp. 25°C, UV detection 254 nm.

Cyclodextrin	none	α	β	γ	HP-α	HP-β	HP-γ	Me-β	HE-β
k′ values	81	81	6.12	18.7,20.7	46	6.75, 7.44	45.6	21	31
Rs values	-	0	0	1.06	0	0.3	0	0	0

Further method development proceeded using γ-CD, which clearly demonstrated a higher degree of chiral selectivity than the other CD molecules. The optimum MeCN content was found to be 12%. Below this the k′ values of the enantiomers were approaching 45 or greater, which is unacceptable for routine analysis work, whilst the increase in chiral resolution seen (1.1 at 8% MeCN) was small. The selectivity (α) was quite high (1.11) but the efficiency of the chromatographic system was inherently very low, with typical N values between 2500 and 4500 theoretical plates. Resolution is strongly dependant on the number of such plates, which are generally greater than 10,000 in a standard HPLC set-up. An increase in the number of theoretical plates may have improved the peak separations but the already high k′ values precluded the use of a conventional 250 x 4.6

mm column to accomplish this. Capillary electrophoresis, where large N values are common, provided a solution to this problem (see section 4.4.).

Figure 3.19. Chromatogram of mequitamium in the presence of γ-CD. HPLC conditions: pKb pre-column (20 x 4.6 mm) x2, 12% MeCN:88% $NaH_2Citrate$ (10 mM) containing 12 mmol γ-CD, 0.8 ml/min flow, Temp. 25°C, UV detection 254 nm.



K_f (association constants) and $k'_{a.cd}$ (complex retention values) were determined for each enantiomer from the variation of k′ with mobile phase γ-CD concentration. By plotting k'_{obs} vs $(k'_s - k'_{obs})/(CD)$ as in Figure 3.20., the K_f and $k'_{a.cd}$ may be determined from the inverse slope and y-axis intercept respectively. This is the method proposed by Sybilska *et al.* 1986 and given in equation 3.09 (section 3.1.5.). The calculated values are given in Table 3.11. The relatively large $k'_{a.cd}$ values indicate the high retentivity of the diastereomeric complexes on the stationary phase. The adsorption of diastereomeric complexes is believed to be practically zero when the included guest is

completely immersed in the CD cavity (Botta *et al.* 1988). The adsorption (capacity factor) of γ-CD on a ODS stationary phase has been shown to be close to zero, under reversed phase conditions (Dodziuk *et al.* 1994). These results suggest that a significant portion of the mequitamium structure must be protuding from the γ-CD cavity so that it can still be retained strongly by the pKb pre-columns. The K_f values themselves are not significantly different from each other, indicating that the chiral resolution was more strongly influenced by the large $k'_{a.cd}$ values. Sybilska *et al.* 1986 reported that the resolution of mephenytoin enantiomers arises from the differential adsorption of their diastereomeric complexes on an ODS column.

Figure 3.20. Plot of k'_{obs} vs $(k'_s - k'_{obs})/(\gamma\text{-CD})$ mmol. HPLC conditions as Fig. 3.19.

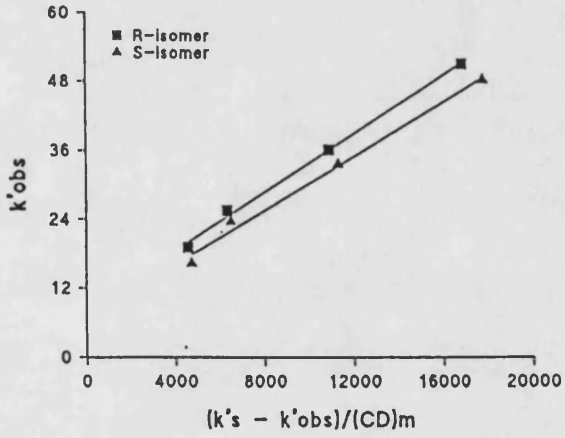


Table 3.11. K_f and $k'_{a.cd}$ values for mequitamium in the presence of γ-CD. HPLC conditions as in Figure 3.19.

Enantiomer	K_f M^{-1}	$k'_{a.cd}$	r value
R-isomer	396	8.5	0.9983
S-isomer	427	6.8	0.9953

3.4.4. Porous Graphitic Carbon (PGC) column.

Although porous graphic carbon (PGC) does not possess any surface functional groups (Kaur 1991), which would make it amenable to the analysis of quaternary nitrogen compounds such as mequitamium, electron rich solutes will be strongly retained due to a unique interaction with the delocalized electron conduction bands of PGC (Bell *et al.* 1994). The π-electron aromatic rings of mequitamium may have been responsible for the strong retention of the analyte seen on the PGC column. High amounts of organic modifier (>60% MeCN and MeOH) were necessary to elute the solute even in the presence of various CD molecules (Table 3.12., see p.84). Such high levels of organic modifier may explain the total absence of any optical resolution in the presence of any of these CDs, as the modifiers out-competed the analyte for the CD cavities.

The overall pattern of k′ factor changes here is similar to that observed when using a standard hydrophobic HPLC column under reversed phase conditions (Table 3.10.), although the extent of the changes here is smaller viz. the high level of organic modifier has lessened the ability of the CDs to complex with the analyte and hence reduce its interaction with the PGC surface. However Me-β-CD caused a much greater relative reduction in analyte retention with the PGC phase than with the pKb columns. This is likely to have been due to the preferential adsorption of this rather hydrophobic CD onto the PGC surface and the subsequent generation of a 'dynamically coated stationary phase', which hindered the interaction of mequitamium with the column surface, thus causing its faster elution. Such 'dynamically coated phases' have been reported before, but only with C18 stationary phases and methylated CDs (Zukowski *et al.* 1989).

Table 3.12. k′ values for mequitamium in the presence of various CDs. HPLC conditions: Na_2HPO_4 (3 mM) - MeCN, (40-60 v/v%), pH 3.1, containing 2.5 mM CD. PGC column Hypercarb S (100 x 4.6 mm), 1 ml/min, UV detection 254 nm. Temp. 25°C.

CD	none	α	β	γ	HP-α	HP-β	HP-γ	Me-β	HE-β
k′ values	97	90	74	66	87	61	81	19	52

3.4.5. Comparison of HPLC Results for Mequitamium.

The only method to separate the mequitamium enantiomers was that using a reversed phase pKb pre-column with the addition of a CD (either γ or HP-β-CD) to the mobile phase (section 3.4.3.). When a PGC column was employed the k′ values increased greatly, from < 21 with the pKb columns to over 60, even in the presence of γ-CD and enantio-recognition was lost. The two aromatic electron rings of mequitamium will have a greater influence on its retention with the PGC surface than on an ODS type phase, due to the possibility of charge transfer and /or π-electron overlap occurring with the delocalized electron bands of PGC (Lim 1992). This explains the longer k′ values found on the PGC column for mequitamium and also accounts for its lack of chiral resolution as the affinity of the enantiomers for the CD molecules in the mobile phase is reduced by their strong adsorption on to the stationary phase surface.

A γ-CD bonded column was unable to provide optical resolution (section 3.4.1.) in contrast to the use of the same chiral selector when dissolved in the mobile phase (section 3.4.3.). Clearly the selectivity of the γ-CD macrocycles towards mequitamium has been adversely affected by tethering the host molecules to the silica stationary phase. Constraints on the CD orientation, alterations to its overall geometry and possible solute interaction with the linking spacer arm, are potential reasons for such observations. Analyte:CD stoichiometric ratios are often 1:1, but higher ratios have been reported

(Hamilton and Chen 1988 and Szejtli 1981). If the stoichiometric ratio was greater than 1:1 with mequitamium (e.g. 2:1 in favour of the γ-CD molecule), it could help to account for the lack of resolution using the bonded phase, as the sterically constrained CD molecules would be less able to form such ratios. However the linear relationship seen in Fig. 3.20. between k'_{obs} *vs* $(k'_s - k'_{obs})/(\gamma\text{-CD})$ is in accord with a 1:1 complex stoichiometry (Allenmark 1991), which suggests that some aspect of the linkage procedure itself is responsble for the lack of resolution. Further, calculation of the $k'_{a.cd}$ values of the enantiomers (section 3.4.3.) showed that the high retention of the diastereomeric adducts on the achiral column surface, was very important to their optical resolution. This behaviour is no longer a factor when employing a CD bonded column and it is not unexpected then, that no resolution should occur because the difference in the R and S enantiomer K_f values was not significant (Table 3.11.). Separations in the polar organic mode were not possible because of the lack of suitable hydrogen bonding / acceptor sites on mequitamium. The criteria for chiral recognition by this means are then perhaps more rigorous than for the other techniques examined, making its general applicability limited.

CHAPTER FOUR

FREE SOLUTION CAPILLARY ELECTROPHORESIS (FSCE) RESULTS AND DISCUSSION

4.1. Introduction.

Electrophoresis can be defined as the differential movement of charged species by attraction or repulsion in an electrical field. As a separation technique it was first introduced by Tiselius in 1937 when he demonstrated the separation of charged proteins in a buffer filled tube. Most later work concentrated on using anti-conductive stabilizers such as gels, in order to try to overcome the inherent problems of sample thermal diffusion and convection (Joule heating), which severely limited efficiency and resolution. However these stabilizers have associated problems such as eddy migration and matrix interactions with the samples (Smith and Evans 1994). Narrow capillaries filled with buffers overcome these problems and the work of Hjertén in 1967 and Mikkers in 1979 led the way in using capillaries with internal diameters of ≤ 1 mm. Jorgenson and Lukacs (1981a and 1981b) pioneered the application of fused silica capillaries with internal diameters of 75 μm (with potential differences around 30 KV) and demonstrated for the first time that very high efficiencies and short analysis times were achievable.

4.1.1. Basic Theory of FSCE.

The mathematical treatment described here is largely based on the theoretical work performed by Jorgenson and Lukacs 1981a and 1981b and Jorgenson 1984. When an electric field is applied across a capillary filled with a buffer (FSCE), anions migrate toward the anode (positive electrode) and cations migrate towards the cathode (negative electrode). The velocity of migration, v, can be given by;

$$v = \mu_{app} E \qquad \text{(Equation 4.01.)}$$

where E is the potential field strength (equal to V, the applied voltage divided by L, the total length of the capillary) and μ_{app} is the apparent electrophoretic mobility.

The time taken, t, for a charged sample to migrate the length of a capillary is given by;

$$t = L^2/\mu_{app}V \qquad \text{(Equation 4.02.)}$$

There are two capillary lengths involved in the separation process, namely L the total length and W the length from the inlet to the detector window, such that equation 4.02. may be expressed more accurately by;

$$t = WL/\mu_{app}V \qquad \text{(Equation 4.03.)}$$

This equation predicts that high voltages and/or short capillaries will provide rapid analysis times. Molecular longitudinal diffusion occurs as the analyte migrates along the capillary producing band broadening (peak variance, σ^2), which may be determined by;

$$\sigma^2 = 2Dt = 2DWL/\mu_{app}V \qquad \text{(Equation 4.04.)}$$

where D is the analyte diffusion coefficient. Radial diffusion (across the capillary) is unimportant due to the plug flow profile caused by electroendosmotic flow (EOF). This is the bulk flow of liquid within the capillary across the inner surface upon application of an electric field, which arises as a consequence of the formation of an electrical double-layer on the interior wall. Ionized silanols (negative charge) attract positively charged buffer components to maintain a charge balance, causing the formation of an electrical potential (the zeta potential, ζ) close to the capillary wall. The cations are strongly hydrated such that upon creating a potential drop across the capillary, both they and the bulk liquid are drawn towards the cathode creating a flat flow profile.

The mobility of EOF, μ_{eo}, (first described by Pretorius *et al.*) may be given as;

$$\mu_{eo} = (\epsilon\zeta E/4\pi\eta) \qquad \text{(Equation 4.05.)}$$

where ϵ is the dielectric constant of the buffer and η is the buffer viscosity. The EOF is strongly pH dependant; at high pH, where the silanols are significanly deprotonated, the flow will be rapid whereas at low pH the EOF will be greatly reduced due to the small zeta potential. In the presence of EOF the apparent elçtrophoretic mobility, μ_{app}, is given by;

$$\mu_{app} = \mu_{ep} + \mu_{eo} = WL/tV \qquad \text{(Equation 4.06.)}$$

The μ_{eo} may be determined by injecting a neutral analyte (eg. acetone, DMSO, MeCN) and measuring the time taken for this sample to reach the detection window and then applying equation 4.06., remembering that the μ_{ep} (the samples electrophoretic mobility) value will be zero.

4.1.2. Operational Modes of Capillary Electrophoresis Utilising CDs.

Electrophoresis encompasses a family of techniques, the main ones being free solution capillary electrophoresis (FSCE), micellar electrokinetic chromatography (MEKC), isoelectric focusing (IEF), capillary gel electrophoresis (CGE) and isotachophoresis (ITP). IEF is used to separate and identify proteins and peptides based on differences in their isoelectric points (pI). It is not of benefit to add cyclodextrins to the ampholytes and so IEF has not been employed for chiral work. However all the other modes have been used to provide chiral separations with cyclodextrins, examples of which are given in the following text.

4.1.3. Isotachophoresis (ITP).

ITP can be used to separate samples that contain either positively or negatively charged species. The mixture to be separated is introduced between the so-called leading (LE) and terminating electrolytes (TE). The LE contains an ion with a higher effective mobility than that of any of the sample ions whilst the TE contains an ion with a lower effective mobility than any sample ion. Upon application of an electric field migration proceeds until a steady state is achieved, with the sample separated into distinct zones, which migrate in close contact with one another at the same velocity.

In the first sucessful report of optical resolution in electrophoresis, Snopek *et al.* 1988 examined a series of ephedrine related alkaloids with β-CD derivatives by ITP and found that separation depended upon using an acidic pH of 5 (at pH 9.7 no resolution occured). Shortly thereafter, Smolková-Keulemansová 1988 used α-CD in the LE to separate positional isomers of halogen substituted benzoate anions along with β- and dimethyl-β-CD for the chiral resolution of pseudoephedrine and related alkaloids. Phenothiazine and some allied drugs were achirally resolved by Snopek *et al.* 1989 using trimethyl-β-CD, whilst γ- and β-CD proved effective for the chiral resolution of some of these test solutes. Tanaka *et al.* 1991 employed β-CD in the LE to resolve the enantiomers of several catecholamines and found that addition of a neutral surfactant affected resolution.

4.1.4. Micellar Electrokinetic Chromatography (MEKC).

MEKC was first introduced by Terabe *et al.* 1984 for the resolution of neutral phenol derivatives, where the separation was based on solute partitioning between a pseudo-stationary phase consisting of micelles (Sodium dodecylsulphate, SDS) and the bulk liquid phase. The technique allows for the separation of neutral and charged solutes at

the same time. Partitioning into the micelles is affected by solute size, hydrophobicity and charge (where the micelle itself is charged eg. SDS, Hexadecyltrimethylammonium bromide [HTAB], bile salts).

Terabe 1989 and Terabe *et al.* 1990 further modified the MEKC separation mechanism with the addition of CDs. They were able to resolve a series of chlorinated benzene congeners and tetrachlorodibenzodioxin isomers using γ-CD and SDS in the buffer. The CDs are not believed to interact with the micelles due to their hydrophilic exterior but induce an additional solute complexation equilibrium with the sample moving between the CD, micelle and the bulk solution. Nishi *et al.* 1991, Soini *et al.* 1992 and Nussbaum *et al.* 1994 have all used this method to enantiomerically resolve barbiturates, NSAID's and other pharmaceuticals using a variety of CDs and micelles. The basic equation for MEKC, as derived by Terabe *et al* 1984 is;

$$k' = t_{Rm} - t_0 / t_0[1-t_{Rm}/t_{mc}]$$

where k' is the 'capacity factor', t_{Rm} is the solute retention time, t_0 is the retention time of a solute with no interaction with the micelles and t_{mc} is the retention time of a solute completely solubilised by the micelles.

4.1.5. Capillary Gel Electrophoresis (CGE).

CGE is mainly associated with separation based on differences in solute size as analytes pass through the pores of a gel matrix which acts as a 'molecular sieve'. Larger solutes are hindered more than smaller ones with the result that they migrate more slowly. Traditionally the technique has been used to separate proteins and DNA fragments. However the addition of CDs to the matrix may allow chiral separations to occur.

Guttman *et al.* 1988 demonstrated the separation of a range of dansyl amino acids using a polyacrylamide gel containing α-, β- or γ-CD. β-CD gave the best chiral resolution values for these analytes. Swartz 1993 reported the use of derivatized CDs incorporated into similar gels for the resolution of both acidic and basic small pharmaceuticals. Cruzado and Vigh 1992 synthesised allyl carbamloy derivatised β-CD and polymerised this with acrylamide to form CD containing gels, which were used to resolve the enantiomers of homatropine and dansyl amino acids.

4.1.6. Free Solution Capillary Electrophoresis (FSCE).

This is the most widely employed electrophoretic technique due mostly to the ease and speed with which an analysis can be performed. Buffer compositions are inherently more simple than the other methods and the fused silica capillaries required are relatively cheap and readily available so that optimization is more tractable. The underlying theory was discussed in section 4.1.1. and the following text details some related work in the field with CDs.

Fanali 1989 was among the first to describe chiral resolution in FSCE using β-CD and methylated derivatives to separate the enantiomers of some sympathomimetic drugs. He extended this work using other analytes and investigated the affect of methanol, urea and temperature on resolution (Fanali 1991 and Schutzner and Fanali 1992). Snopek *et al.* 1991 added alkylhydroxyalkycellulose derivatives to buffers containing CDs and found that it resulted in improvements in the resolution of chloramphenicol and thioridazine enantiomers. Quang and Khaledi 1993 used tetraalkylammonium reagents in conjunction with various CDs to improve the chiral separation of a group of basic pharmaceuticals. They suggested this occurred due to capillary wall coverage by the reagents preventing analyte interaction with the negative surface layer, so improving efficiency and hence

observable resolution. Hjerten 1985 coated the inner silica surface with a neutral polymer to eliminate EOF and was able to separate a group of aromatic carboxylic acids which could not be done with an uncoated tube. Towns and Regnier 1991 prepared nonionic surfactant coated capillaries for the separation of proteins, which allowed the pH to be altered (to improve selectivity) without affecting the flow rate.

A huge range of CDs have been tested in FSCE to effect novel separations. A charged CD, (2-*O*-carboxymethyl-β-CD) was used by Terabe *et al.* 1985 to resolve positional isomers of substituted benzenes, Sepaniak *et al.* 1992 used glycosylated α-CD to separate phosphorylated binaphthyl enantiomers and Nardi *et al.* 1993 resolved some 2-hydroxy acids with 6-methylamino-β-CD. Other CDs that have been employed for chiral resolutions include γ and hydroxypropyl-β-CD (Copper *et al.* 1994); α-, β-, dimethyl-β and hydroxypropyl-β-CD (Pálmarsdóttir and Edhlom 1994); hydroxyethyl-β-CD and methyl-β-CD (Peterson 1993); sulfobutyl ether β-CD (Chankvetadze *et al.* 1994); hydroxypropyl-α-CD and hydroxypropyl-γ-CD (Rogan *et al.* 1994), permethylated α- and β-CDs (Valkó *et al.* 1994) and a carboxymethylated-β-CD polymer (Aturki and Fanali 1994).

4.1.7. Equilibria in FSCE upon the Addition of CDs.

Wren and Rowe 1992 were the first to propose a simple mathematical model for FSCE which described the behaviour of a solute when in contact with a chiral agent added to the buffer. They subsequently went on to substantiate this model with an investigation into the interaction between some β-blockers and methyl-β-CD (Wren and Rowe 1993).

When an analyte enantiomer, A, interacts with a chiral selector, C, the following equilibrium occurs;

$$\begin{array}{ccc} & \overset{K_1}{} & \\ A + C & \rightleftharpoons & AC \\ \downarrow & & \downarrow \\ \mu_1 & & \mu_2 \end{array}$$ (Equation 4.07.)

where μ_1 is the electrophoretic mobility of the analyte enantiomer in free solution, μ_2 is the electrophoretic mobility of the analyte-chiral selector complex and K_1 is the equilibrium constant. A second equilibria, K_2, exists for the other analyte enantiomer and if these two equilibrium constants are different (i.e. the enantiomers have different affinities for the chiral selector) and the mobilities μ_1 and μ_2 are also different, then chiral resolution may occur.

The apparent electrophoretic mobility of A, μ_a, will be a function of the time it spends as the free solute and in a complexed form;

$$\mu_a = \{[A] / [A] + [AC]\}\, \mu_1 + \{[AC] / [A] + [AC]\}\, \mu_2 \quad \text{(Equation 4.08.)}$$

$$[AC] = K_1[A][C] \quad \text{(Equation 4.09.)}$$

hence,

$$\mu_a = \mu_1 + \mu_2 K_1[C] / 1 + K_1[C] \quad \text{(Equation 4.10.)}$$

The difference in apparent electrophoretic mobilities of the two enantiomers can then be given by;

$$\Delta\mu_a = [C](\mu_1 - \mu_2)(K_2 - K_1) \;/\; 1 + [C](K_1+K_2) + K_1K_2[C]^2 \quad \text{(Equation 4.11.)}$$

Mobility differences (and thus resolution) will be zero if one of the following holds, $K_1 = K_2$, $\mu_1 = \mu_2$, [C] = 0 or is very large. Between these two extremes of [C] lies some

value which will produce the optimum mobility difference and provide good resolution. Penn *et al.* 1993 developed a similar equation to equation 4.11. and showed (via differential calculus of equation 4.11.) that the maximum mobility difference occurs when $\Delta\mu_a / \mu_a[C] = 0$. This condition is fulfilled when;

$$K_{av}[C] = 1 \qquad \text{(Equation 4.12.)}$$

where K_{av} is the average binding constant. Furthermore the maximum resolution of the enantiomers will occur when;

$$K_{av}[C] = [(\mu_{eo} + \mu_0)/(\mu_{eo} + \mu_\infty)]^{½} \qquad \text{(Equation 4.13.)}$$

where μ_{eo} is the EOF rate, μ_0 is the mobility of the free analyte and μ_∞ is the electrophoretic mobility of the analyte-chiral selector complex. Penn *et al.* 1993 investigated the interaction of tioconazole enantiomers with hydroxypropyl-β-CD by FSCE and found good agreement between their actual and predicted results.

Resolution in FSCE can be expressed according to Giddings 1969, by;

$$R_s = ¼\ N^{½} \cdot \Delta\mu/\mu \qquad \text{(Equation 4.14.)}$$

where $\Delta\mu$ is the difference in electrophoretic mobilities of two analytes (enantiomers), μ is their average electrophoretic mobility and N is the number of theoretical plates given by;

$$N = \mu VW/2DL \qquad \text{(Equation 4.15.)}$$

Combination of equations 4.11. and 4.14. gives a general expression for the resolution of enantiomers with CDs (Pálmarsdóttir and Edholm 1994);

$$R_s = \frac{1}{4} N^{\frac{1}{2}} \cdot [C](K_2 - K_1)(\mu_1 - \mu_2)/\mu_1 + \frac{1}{2} (K_1 + K_2)(\mu_1 + \mu_2)[C] + \mu_2 K_1 K_2 [C]^2$$

(Equation 4.16.)

However this equation is too unwieldy to be applied generally and resolution is often calculated by;

$$Rs = 1.177 \cdot (t_2 - t_1)/(W_{a\frac{1}{2}} + W_{b\frac{1}{2}})$$ (Equation 4.17.)

where $W_{a\frac{1}{2}}$ is the peak width at half height for the first migrating enantiomer (Wren and Rowe 1992).

4.1.8. Experimental Parameters Affecting Separation in FSCE.

EOF, which is strongly pH dependant, can prove detrimental to resolution by 'sweeping' the analytes along at such a rate as to overcome their individual electrophoretic mobilities. Terabe *et al.* 1988 developed an equation to describe resolution, including a term for EOF, μ_{eo};

$$R_s = \frac{1}{4} (V/32D)^{\frac{1}{2}} \cdot (W/L)^{\frac{1}{2}} \cdot \Delta\mu_{ep}/(\mu_{ep} + \mu_{eo})^{\frac{1}{2}}$$ (Equation 4.18.)

Increasing the EOF will then reduce R_s therefore it is necessary to control pH to maintain a high resolution, whilst having migration times that are short enough to be of practical use.

Increasing the applied voltage will lower solute migration times according to equation 4.03. and improve R_s according to equation 4.18. but there is a practical limit to the voltage strength caused by Joule heating (Ohmic resistance of the buffer to electrical flow). If the buffer temperature rises beyond the heat dissipation capabilities of the capillary, then band-broadening will occur due to thermal convection altering the buffer viscosity. Hence narrow capillaries with good thermal dissipation properties are favoured. Short capillaries will provide rapid analysis times according to equation 4.03. but lower R_s according to equation 4.18. Therefore some compromise is required between the overall capillary dimensions and the applied voltage to provide adequate analysis conditions.

Buffer concentration and ionic strength are related to EOF by equation 4.05. whereby an increase in concentration will increase the viscosity, η, and so reduce EOF. With narrow capillaries ($\leq$ 50 mm) migration times will increase as the buffer ionic strength is increased. The pH should be within ± 2 pH units of the buffer pKa in order to maintain effective pH control and thus consistent migration times. Generally speaking, buffers of low conductivity are preferred as they allow high voltages (and thus rapid separations) with minimum temperature rises inside the capillary (Bello *et al.* 1992).

Organic solvents are often added in FSCE buffers where they can act as solubilizing agents and modifiers of EOF. Alcohols tend to decrease EOF whereas acetonitrile often causes a small increase (Weinberger 1991). Fujiwara and Honda 1987 also found that the alcohol methanol increased solute migration times whilst acetonitrile lowered them (i.e. faster migration times). Wren and Rowe 1992 suggested that organic modifiers could either increase or decrease analyte enantio-resolution values (in the presence of CDs) depending upon the concentration of CD in the buffer. An examination of propranolol with β- and methyl-β-CD supported their proposals.

4.2. CE Investigation of Phenethylamines.

The resolution of nine sympathomimetic phenethylamine racemates (see Fig. 3.2. on p. 49 for their structures) by β-CD and *heptakis*(2,3-di-*O*-acetyl)β-cyclodextrin (Ac-β-CD) was investigated by capillary electrophoresis. Only β-CD and peracetyl β-CD had provided any chiral resolution for this group of analytes by HPLC (section 3.2.). It was found impossible to use peracetyl β-CD in the CE buffers as the required levels of organic modifier (for CD dissolution) caused problems with consistent current generation. The addition of urea did not improve CD solubility. Consequently the related 2,3-di-*O*-acetyl derivative, which is more water soluble, was examined.

The resolution of some phenethylamines by CE has been reported previously using cyclodextrins. Fanali 1989 resolved the enantiomers of ephedrine, isoproterenol (isoprenaline) and noradrenaline using di-*O*-methyl-β-CD but could not repeat this with native β-CD. Nielen 1993 was able to determine a 100-fold excess of (+)-ephedrine in the presence of the (-) isomer, also using di-*O*-methyl-β-CD, but not β-CD. Dette *et al.* 1994 used an anionic β-CD derivative to chirally resolve ephedrine and found that it gave better results than β-CD itself. Rogan *et al.* 1994 separated salbutamol enantiomers with dimethyl-β-CD, calculating that they possessed a weak binding constant with the CD. Bechet *et al.* 1994 resolved ephedrine and isoprenaline with a range of CD types, Heuermann and Blaschke 1993 resolved oxedrine (amongst others) with Me-β-CD and Chankvetadze *et al.* 1994, employing a charged β-CD derivative, separated the enantiomers of etilefrine.

Data for the chiral discrimination of a range of phenethylamines by β-CD and the previously untried Ac-β-CD are reported here.

4.2.1. Complexation with Various CDs.

α-, HP-α and γ-CD were found to have little effect on the migration times of the analytes and did not produce any chiral resolution. The migration times for all nine compounds using 0.1M phosphate buffer pH 3.0 are shown in Table 4.1., which also gives the migration times (t_m) and resolution values (R_s) measured in the presence of β-CD or Ac-β-CD.

Table 4.1. CE migration times (minutes) and resolution values (R_s) for some phenethylamines. CE conditions: 0.1M phosphate buffer pH 3.0 with and without 12 mM β-CD or Ac-β-CD. Coated-capillary (20 cm x 25 μm). Samples were loaded by electromigration and separated at room temperature using a constant current of 12 μA.

Compound	buffer alone (t_m)	with β-CD		with Ac-β-CD	
		(t_{m1},t_{m2})	R_s	(t_{m1},t_{m2})	R_s
ephedrine	5.39	9.98	0	6.66,6.87	1.25
oxedrine	8.98	10.91	0	7.55,7.93	1.62
oxilofrine	6.09	12.33,12.71	1.16	6.32,7.97	1.74
norfenefrine	8.36	11.95	0	7.43,7.65	1.31
etilefrine	9.49	10.92,11.11	0.47	8.84.9.82	4.28
orciprenaline	7.12	12.61,12.94	0.89	9.22,9.48	1.32
noradrenaline	9.39	11.80	0	7.55	0
isoprenaline	11.10	13.84	0	9.24	0
salbutamol	12.18	12.74	0	9.27,9.45	0.85

The migration times in Table 4.1. correspond to electrophoretic mobilities ranging from 0.58×10^{-4} (t_m = 13.84 min) to 1.72×10^{-4} (t_m = 5.39 min) $cm^2 s^{-1} V^{-1}$, which were calculated according to equation 4.06 (see section 4.1.1.).

In an attempt to measure μ_{eo} under the experimental conditions used, acetone was employed as a neutral marker during a time of 90 minutes over a column distance of 2.8 cm. No response for acetone was obtained. This indicated that μ_{eo} must be less than $1.8x10^{-6} cm^2 s^{-1} V^{-1}$ which is lower than that in a previous report (Wren and Rowe 1993 refU), where the effect of μ_{eo} was discounted at $0.04x10^{-4} cm^2 s^{-1} V^{-1}$. Thus the endo-osmotic flow, which is minimised when using coated capillary columns, can be ignored so that $\mu_{ep} = \mu_{app}$.

β-CD increased the migration times for all compounds relative to the uncomplexed drug whereas Ac-β-CD caused a decrease in migration except for three compounds (ephedrine, oxilofrine and orciprenaline). The increase in migration times for these three compounds with the derivatised CD was less than that observed with β-CD and thus permitted more rapid analysis. β-CD resolved only three compounds (oxilofrine, etilefrine and orciprenaline) and the acetylated CD increased the resolution of all racemates compared to the underivatised oligosaccharide, except for noradrenaline and isoprenaline, which were not resolved by either CD. Individual enantiomers of the compounds were not available so the elution order of the R- and S-isomers is not known, with the exception of ephedrine, where the (+)-enantiomer was found to migrate fastest i.e. it had a weaker complexation with the CD molecule.

The effect of buffer pH on migration times and resolution was fully investigated for all compounds in the presence of Ac-β-CD. Migration times at pH 3.0, 4.5, 6.0 and 7.5 are given in Table 4.2., while Figure 4.1., on p.101, shows the effect of pH on resolution. Generally, as the pH was raised migration times increased and resolution decreased, although with etilefrine the resolution showed a minimum at pH 6.0.

The effect of adding 5, 10, 15 and 20%v/v acetonitrile to the 0.1M phosphate buffer at pH 3.0 was investigated for three compounds (ephedrine, oxilofrine and orciprenaline). Migration decreased to a minimum at 15%v/v and then increased again at 20%v/v.

Resolution decreased with increasing concentration of the organic modifier and was accompanied by deterioration of peak shape.

Figure 4.1. Influence of pH on resolution (R_s) of various racemic phenethylamines. CE conditions, 100 mM phosphate buffer pH 3.0 in the presence of 12 mM Ac-β-CD. Other conditions as in Table 4.1.

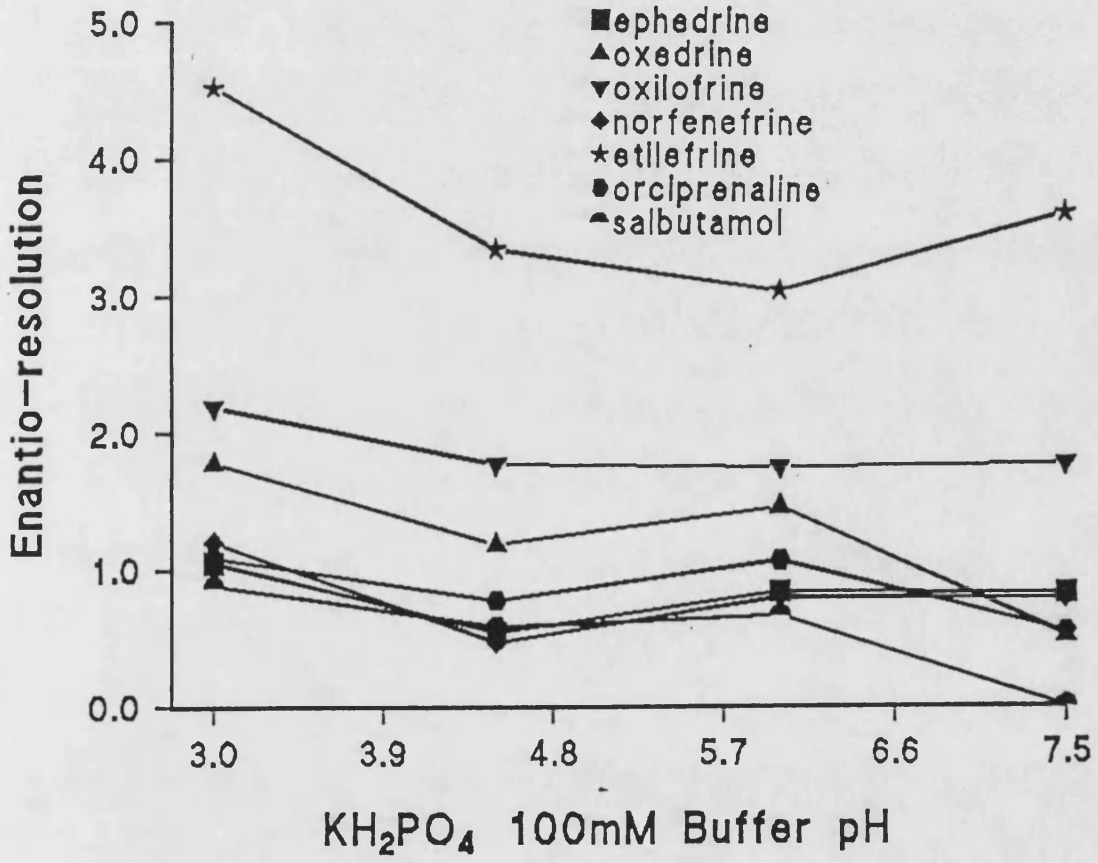


Table 4.2. Effect of pH on CE migration times (minutes) for the phenethylamines. CE conditions, phosphate buffer (0.1M, pH 3.0) in the presence of Ac-β-CD. Other conditions as in Table 4.1. pKa values are approximately 8 - 9.

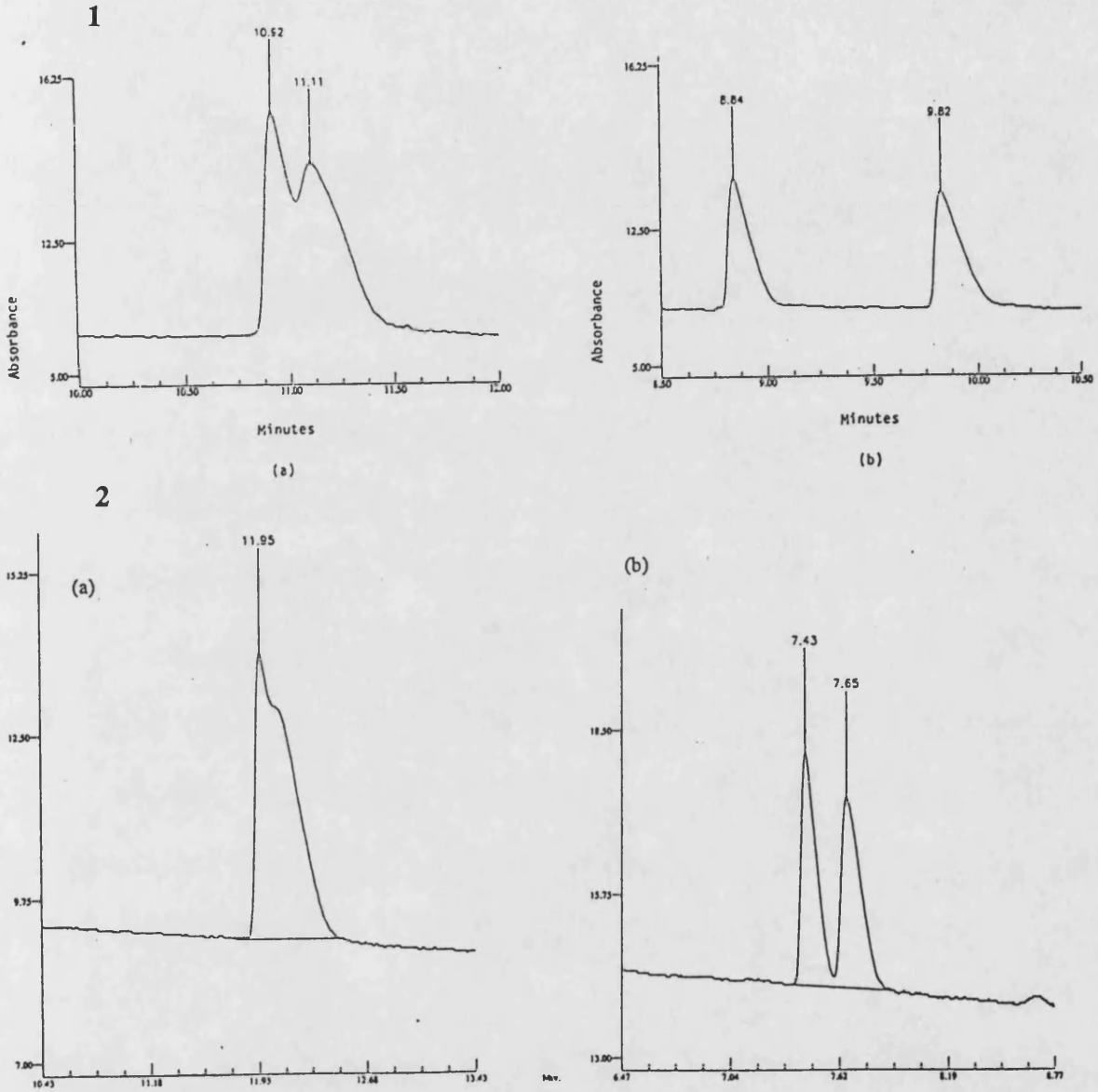
Compound	pH 3.0	pH 4.5	pH 6.0	pH 7.5
ephedrine	6.66, 6.87	7.18, 7.35	8.04, 8.24	12.77, 13.17
oxedrine	7.55, 7.93	7.70, 7.97	10.30, 10.66	14.40, 14.81
oxilofrine	7.97, 8.32	8.12, 8.47	9.13, 9.48	15.94, 16.65
norfenefrine	7.43, 7.56	7.19, 7.30	9.06, 9.23	15.00, 15.34
etilefrine	8.84, 9.82	8.62, 9.26	10.40, 11.10	16.93, 18.42
orciprenaline	9.22, 9.48	9.73, 10.00	11.20, 11.49	19.12, 19.54
noradrenaline	7.55	7.71	10.08	18.81
isoprenaline	9.24	9.53	13.96	20.82
salbutamol	9.27, 9.45	9.97, 10.10	13.03, 13.22	16.41

4.2.2. Comparison of Results.

The phenethylamines discussed have amine pK_a values in the range 8.6 to 9.3, and thus under the conditions used here for CE, they are expected to be fully protonated and migrate to the cathode. β-CD caused an increase in migration time consistent with complexation reducing the mobility of the drug (Table 4.1.). Ac-β-CD complexes migrated more quickly than β-cyclodextrin complexes with all the phenethylamines studied, which could perhaps be explained by the formation constants being smaller for the derivatised cyclodextrin. Unexpectedly, however, Ac-β-CD also caused a decrease in migration times relative to the uncomplexed molecule for six of the nine compounds which is difficult to explain. Although migration times were faster with the derivatised CD, seven of the nine compounds were resolved compared with only three for β-CD.

Figure 4.2. Electropherograms of (1) etilefrine HCl and (2) norfenefrine HCl.

With the addition of (a) β-CD and (b) Ac-β-CD. CE conditions as in Table 4.1.



Overall, increasing pH caused an increase in migration times as would be expected by increasing the proportion of neutral phenethylamine species in the buffer. Addition of up to 15% MeCN to the buffer in the presence of β-CD decreased mobility for the three compounds tested (ephedrine, oxilofrine and orciprenaline) perhaps by causing an increase in the affinity of the analytes for the bulk solvent, which migrates very slowly under these conditions of low electroendosmosis. It was not added to the buffer in the

final conditions chosen because it lengthened analysis time and gave poorer resolution and peak shape.

On the basis of plots of migration times *versus* CD concentration, Fanali 1989 suggested that ephedrine fits into the oligosaccharide cavity better than compounds with a substituted phenyl ring, while the catecholamine enantiomers show greater chiral discrimination. There is no evidence in the CE studies reported here to support this assertion as ephedrine does not show atypical behaviour.

4.2.3. Analyte Structure Relative to Chiral Discrimination.

Noradrenaline and isoprenaline were the only analytes not resolved by either CD type. Also the analyte pairs norfenefrine (R_s = 1.31) / noradrenaline (R_s= 0) and orciprenaline (R_s= 0.89 β-CD,R_s = 1.32 Ac-β-CD) / isoprenaline (R_s= 0) have respectively, identical R groups but different OH substitution patterns of their aromatic OH groups. The unique position of these groups may have sterically hindered their chiral interaction with the CDs. Norfenefrine and etilefrine differed only in the R_2 position, with the ethyl group of etilefrine proving more favourable for enantio-recognition (R_s = 4.28) than the solitary hydrogen on norfenefrine (R_s = 1.31). Comparison of the CE data for oxedrine and oxilofrine, which contrast only by the 2-Me group in oxilofrine, shows that the methyl group is required for resolution by β-CD. Although ephedrine also has the 2-Me group, it was not resolved and this may be due to the lack of a hydroxy substituent on the aromatic ring. Differences in hydrogen bonding patterns could explain why Ac-β-CD (a hydrogen bond acceptor) is better than β-CD (a hydrogen bond donor/acceptor) at discriminating between phenethylamine enantiomers.

Rogan *et al.* 1994 and Heuermann and Blaschke 1993 found that substituted phenyls, such as the phenethylamines, did not complex strongly (if at all) with the rather small α-

CD cavity. Steric constraints and possibly the weak dipole moment of α-CD (Sherrod 1992) have been suggested as reasons for such behaviour (Gelb *et al.* 1981). In the other extreme, γ-CD cavity is much larger than these analytes and any inclusion would not be sufficiently 'tight' to allow retardation of solute migration or provide for chiral recognition. Belder and Schomburg 1994 observed that if the aromatic ring of an analyte had sufficient bulky substituents, then it would be retained and possibly chirally resolved by γ-CD. These results are consistent with a 'size inclusion' model influencing complexation, where the 'tightness of fit' between an analyte and a CD may determine enantio-resolution.

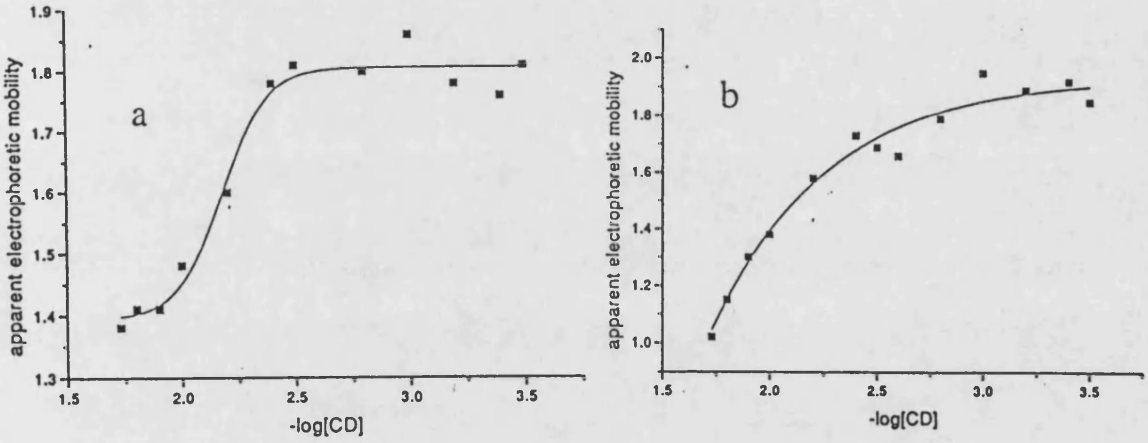
4.2.4. Determination of Complex Formation Constants (K_f).

Gareil *et al.* 1993 have developed a method for determining complex association constants (K_f) between CDs and guest molecules in CE. They were able to calculate the binding between retinoic acid and Me-β-CD from the abscissa of the point of inflection on a graph of μ_{app} *versus* -log [CD]. Here, measurement of the K_f value of etilefrine with β-CD and Ac-β-CD was attempted by a similar means.

The results are shown in Figures 4.3a and b. Each measurement was performed in triplicate and the calculated average K_f value found for etilefrine with β-CD was $155M^{-1}$, but it was not possible to estimate its binding with Ac-β-CD, due to the absence of a lower plateau region on the graph, where the analyte is bound by the CD. The low aqueous solubility of Ac-β-CD prevented the dissolution of any higher concentrations. The shape of the curve in Fig. 4.3b approximates to that region where the analyte has yet to be fully complexed by the chiral selector. This strongly suggests that the analyte forms a weaker complex with the acetylated β-CD, although the actual K_f value was not measurable. This view is supported by examination of etilefrine's migration times (Table 4.1.), which are fastest in the presence of Ac-β-CD, again implying a weaker

complexation constant. In view of the fact that all the phenethylamines possess faster migration times with Ac-β-CD it seems likely that they too have weaker complexation constants than with β-CD.

Figure 4.3. μ_{app} *versus* -log [CD], **(a)** etilefrine and β-CD, **(b)** etilefrine and Ac-β-CD.



4.3. CE Investigation of Propranolol Analogues.

In this work propranolol (**1** in Fig. 3.3.) and related compounds were used to study how changes in molecular structure can affect the complexation process with the various CD's which had provided promising results from the HPLC studies i.e. β-CD, HE-β-CD and Me-β-CD. *Heptakis*(2,3-di-*O*-Acetyl)β-cyclodextrin (Ac-β-CD) was also employed in the CE aqueous buffers, as the peracetylated derivative (water insoluble) had resolved compounds **4** and **5** using the PGC column (3.2.4.). Compounds **2** and **3** are *t*-butyl and ethyl analogues of **1**. Compounds **4** and **5** are *i*-propyl and *t*-butyl analogues of **1** respectively, but with a phenyl ring - not a naphthyl group. Compound **6** (with a naphthyl group) has a unique urea-ethyl linkage, which proved impossible to examine by CE as the weakly basic urea group is not readily available for protonation. Coupled with

its high molecular weight, this resulted in impractical migration times, often greater than one and a half hours.

The aqueous/buffer mixture was chosen to ensure a pH (3.0) at which analytes **1-5** possessed a positive charge (pKa's of compounds **1-5** are around 9 ± 0.5) and thus migrated towards the cathode. Attempts to measure the EOF under these conditions were successful only in that they gave a maximum possible value of 8×10^{-6} cm^2 V^{-1} s^{-1}. This is very low and compares favourably with Wren and Rowe 1993, who ignored EOF at the higher value of 0.04×10^{-4} cm^2 V^{-1} s^{-1}.

4.3.1. Complexation of Propranolol Analogues with Ac-β-CD.

Ac-β-CD only resolved the enantiomers of compounds **1** and **2** under these experimental conditions (Table 4.3.). Acetylation increases the hydrophobicity of the CD and changes its hydrogen bonding ability, which often results in low enantio-selectivity (Krustulovic 1989b).

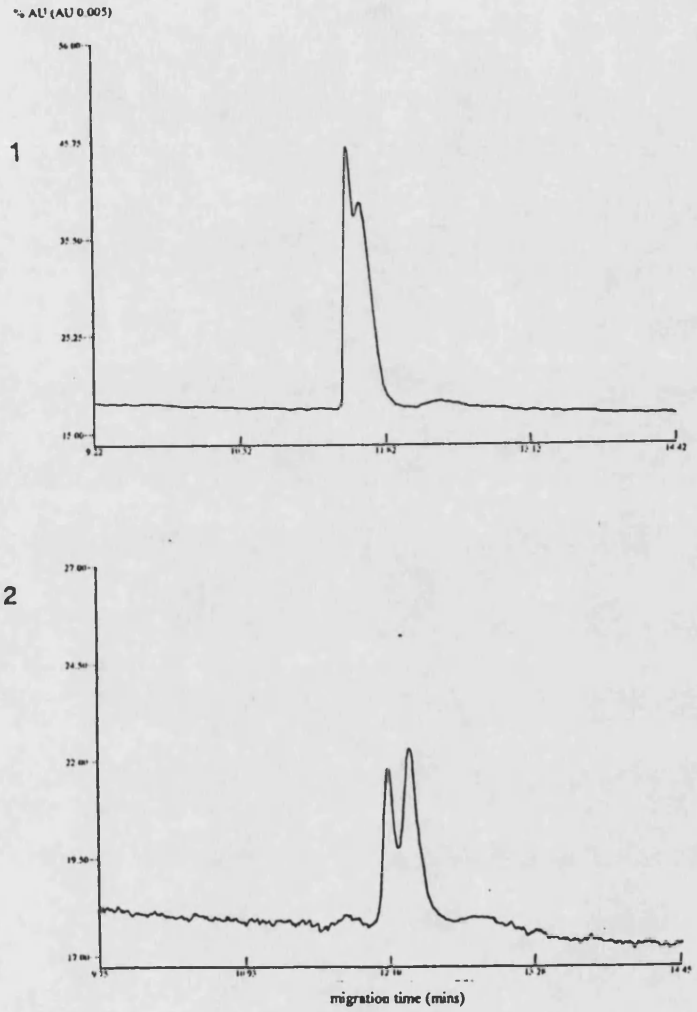
With only the two most hydrophobic compounds (**1** and **2**) showing any enantio-recognition (Fig. 4.4., see p.109) and possessing the longest migration times, it would appear that analyte hydrophobicity is a major factor in the determination of resolution values and complex formation constants when using this CD derivative in FSCE. According to Barnaby and MacLeod 1991 there are few published incidents were Ac-β-CD has provided enhanced separation over the underivatized β-CD. However Yamashoji *et al.* 1992 have shown that *heptakis*(2,3-di-O-Acetyl)β-CD can be used to give improved chiral resolution of DL-alanine β-naphthylamide in CE when compared to β-CD.

Table 4.3. Migration times (t_m, mins) for compounds **1-5** in the presence of increasing amounts (mM) of Ac-β-CD. Resolution values (Rs) in parentheses. FSCE conditions: KH_2PO_4 50mM, pH3, 30% MeOH buffer. Constant current of 5μA. * 4M urea was necessary to enable dissolution of this amount of CD.

Analyte	0.0mM	4mM	7.4mM	15mM	25mM	35mM *
1	6.84 (0)	10.82 (0)	10.88 (0)	11.56,11.65 (<0.1)	11.78,11.89 (0.26)	8.48 (0)
2	6.94 (0)	11.07 (0)	11.35,11.39 (<0.1)	12.27,12.41 (0.63)	12.39,12.56 (0.81)	8.98 (0)
3	6.32 (0)	10.58 (0)	10.67 (0)	11.29 (0)	11.37 (0)	8.24 (0)
4	4.30 (0)	9.82 (0)	10.02 (0)	10.34 (0)	10.39 (0)	6.53 (0)
5	5.59 (0)	10.27 (0)	10.43 (0)	10.86 (0)	11.05 (0)	7.51 (0)

All five analytes however showed longer migration times as the concentration of Ac-β-CD was increased, indicating that they underwent increasing complexation. Due to the insoluble nature of Ac-β-CD it was necessary to use 4M urea to solubilise 35 mM in the buffer. This resulted in lower migration times than would have been expected if urea was absent, and also in the removal of all signs of enantio-recognition for compounds **1** and **2**. The strongly polar urea molecules may have significantly reduced any possible hydrogen-bonding of the CD with compounds **1** and **2**, thus negating resolution and shortening the migration times by increasing the amount of time compounds **1** and **2** spent as faster moving, free analytes.

Figure 4.4. Electropherograms for compounds **1** and **2** in the presence of 25 mM Ac-β-CD. FSCE conditions: 30% MeOH - KH_2PO_4 50 mM, pH 2.5, constant current of 5 μA.



4.3.2. Complexation with Me-β-CD.

Methylated β-CDs have been extensively employed in CE chiral separations, often demonstrating enhanced resolutions over the parent macrocycle (Nielen 1993, Soini *et al.* 1992, Rogan *et al.* 1994, Heuermann and Blaschke 1993 and Gareil *et al.* 1993). Wren and Rowe 1993 stated that in the presence of Me-β-CD, hydrophobicity would be the major force driving complexation in a series of β-blockers. More hydrophobic

solutes would require the presence of less CD to achieve their individual optimum resolution values. This hypothesis is partly supported by these results.

Compounds **1** and **2** each gave maximum resolution values at 25 mM Me-β-CD, with the more hydrophobic compound **2** having a higher resolution value of 0.73, compared with compound **1** which is less non-polar and gave a lower resolution of 0.54 (see Table 4.4., see p.111). Compound **3** is less hydrophobic than either **1** or **2** and gave a lower maximum resolution of 0.32 at the highest Me-β-CD concentration of 35 mM (see Fig. 4.5., see p.112). Similar in behaviour was compound **5** which is less hydrophobic than compounds **1**, **2** or **3** and also gave its maximum resolution at 35 mM. However the resolution of compound **5** (1.54) was higher than observed with the other more non-polar analytes. Also, the least hydrophobic of the compounds, **4**, unexpectedly showed its maximum resolution of 0.68 at only 25 mM Me-β-CD, when it would have been thought to require a higher concentration than the other compounds, based on the simple model advanced by Wren and Rowe 1993.

Different structural features of the five analytes therefore seem to be important not only in the actual complexation process but also in the overall enantio-recognition mechanism. Compounds **4** and **5** gave higher maximum resolution values than compounds **1** and **2** respectively, suggesting that the presence of the naphthyl moiety proved detrimental to resolution by perhaps holding the stereogenic centre too far from the CD rim to permit optimum hydrogen-bonding with the CD hydroxyl groups.

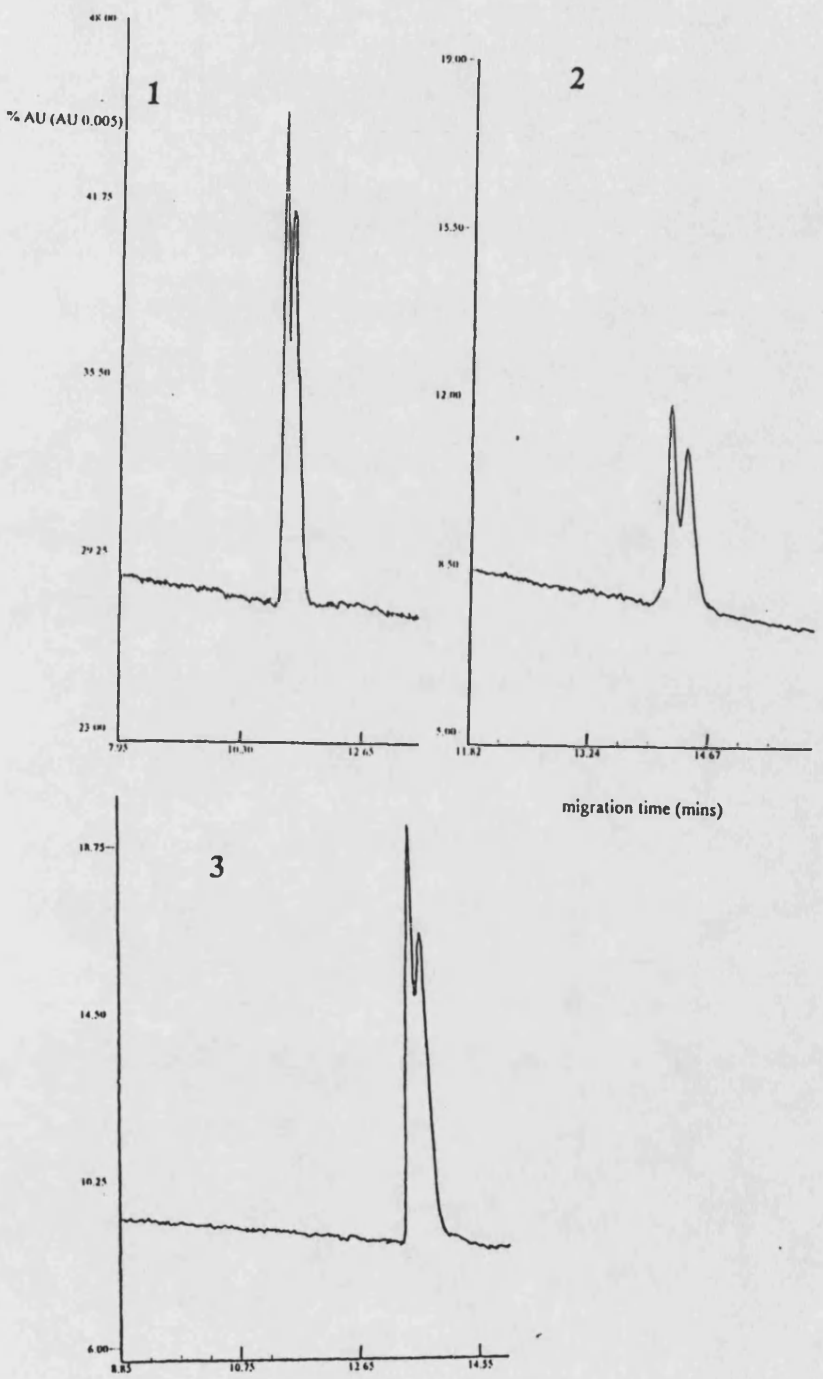
The bulky *t*-butyl R group promoted enantio-recognition in comparison to the *i*-propyl R group with maximum resolution values of **2** > **1** and **5** > **4**. This may be related to the proposed hydrophobicity effect, with the more non-polar compounds having a stronger association with the Me-β-CD and hence also displaying longer migration times.

Table 4.4. Migration times (t_m, mins) for compounds 1-5 in the presence of increasing amounts (mM) of Me-β-CD. Resolution values (Rs) in parentheses. FSCE conditions: KH_2PO_4 50 mM, pH3, 30% MeOH buffer. Constant current of 5 μA.

Compound	0.0 mM	4m M	7.4mM	15mM	25mM	35mM
1	6.84 (0)	7.84 (0)	8.39,8.48 (0.22)	9.37,9.51 (0.26)	11.07,11.23 (0.54)	11.87,12.05 (0.48)
2	6.94 (0)	8.12 (0)	9.37,9.42 (0.17)	10.12,12.23 (0.27)	14.20,14.39 (0.73)	14.34,14.45 (0.31)
3	6.32 (0)	7.50 (0)	7.82,7.88 (0.16)	9.57,9.68 (0.20)	13.44,13.62 (0.30)	14.07,14.18 (0.32)
4	4.30 (0)	6.50 (0)	6.57,6.63 (<0.1)	8.39,8.53 (0.21)	10.90,11.14 (0.68)	11.58,11.82 (0.53)
5	5.59 (0)	7.00 (0)	7.90,7.99 (0.63)	9.08,9.26 (0.73)	11.51,11.78 (1.32)	13.87,14.23 (1.54)

The R groups could interact with the modified CD rim to influence enantio-recognition, perhaps via some steric and/or hydrophobic effect, which alters the analyte's position in the CD cavity and by doing so, holds the analyte hydroxyl and amine groups in a more favourable orientation to encourage chiral recognition.

Figure 4.5. Electropherograms for Compounds 1, 2 and 3 in the presence of Me-β-CD at 25 mM, 25 mM and 35 mM respectively. FSCE conditions: 30% MeOH - KH_2PO_4 50 mM, pH 2.5. Constant current of 5μA.



4.3.3. Complexation with HE-β-CD.

Propranolol (**1**) has been chirally resolved using HE-β-CD in CE (Peterson 1993), although a similar degree of separation with Me-β-CD could not be achieved. In the same work, the author found that HP-β-CD gave equivalent results to HE-β-CD when examining propranolol enantiomers. Other authors have indicated that HE-β-CD generally gave poorer chiral separations than both methylated β-CDs (Rogan *et al.* 1994) and even β-CD itself (Heuermann and Blaschke 1993).

In this work HE-β-CD gave longer migration times for all five compounds than did Me-β-CD or Ac-β-CD, suggesting that the analytes formed stronger inclusion complexes with this CD derivative under the experimental conditions used here. HE-β-CD was also the only CD to provide any enantio-recognition at the 4 mM level, where it partially resolved compounds **1** and **2**, with the more non-polar **2** having a higher resolution value of 0.66.

HE-β-CD caused a reversal in the migration order of compounds **1** and **2** when compared to the results obtained with Me-β-CD and Ac-β-CD. Thus compound **2** had faster migration times than compound **1** over the entire CD concentration range, yet still maintained higher enantio-resolution values than compound **1**. The presence of the analyte naphthyl moiety had a more varied effect on enantio-resolution with HE-β-CD. Although the maximum resolution value of **4** was still greater than that of compound **1** (as with Me-β-CD), compound **5** had a lower maximum resolution than compound **2** which was not the case with Me-β-CD. The most hydrophobic of the solutes i.e. compound **2**, therefore gave the highest individual maximum resolution with HE-β-CD (see Fig. 4.6., p.115) whereas the less non-polar compound **5** displayed the largest resolution with Me-β-CD.

Table 4.5. Migration times (t_m, mins) for compounds 1-5 in the presence of increasing amounts (mM) of HE-β-CD. Resolution values (Rs) in parentheses. FSCE conditions: KH_2PO_4 50 mM, pH3, 30% MeOH buffer. Constant current of 5 μA.

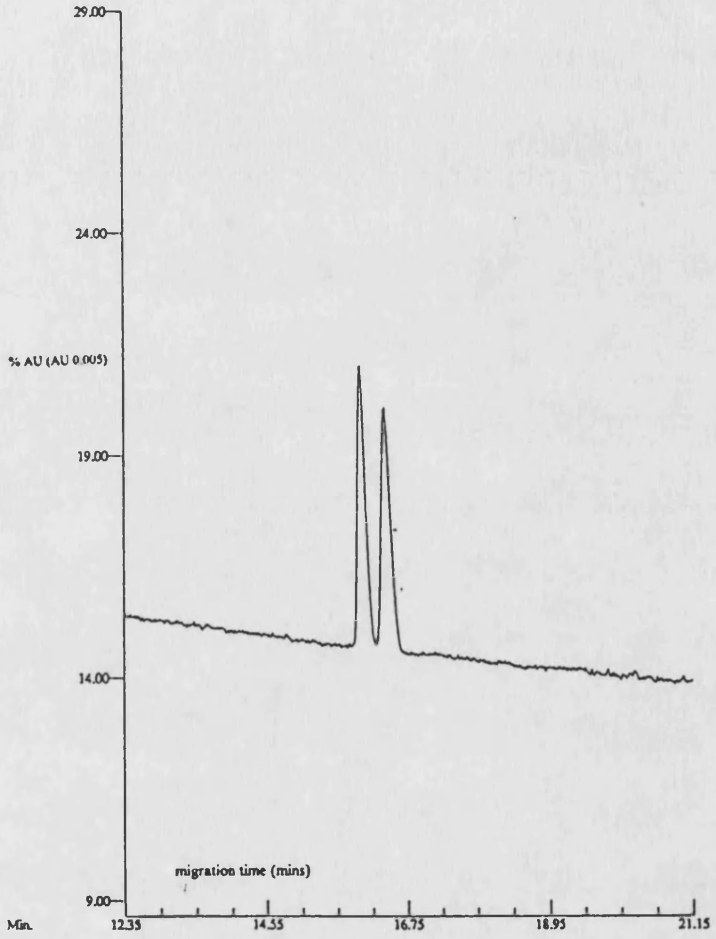
	No CD	4mM	7.4mM	15mM	25mM	35mM
1	6.84 (0)	12.48,12.56 (<0.1)	12.64,12.78 (0.44)	14.39,14.63 (0.74)	15.84,16.12 (0.83)	16.06,16.36 (0.74)
2	6.94 (0)	12.42,12.54 (0.66)	12.53,12.71 (1.08)	13.85,14.14 (1.31)	15.34,15.68 (1.51)	16.00,16.39 (1.67)
3	6.32 (0)	11.19 (0)	11.99,12.07 (<0.1)	13.86,14.01 (0.62)	15.61.15.80 (0.69)	16.63,16.84 (0.77)
4	4.30 (0)	10.10 (0)	10.64,10.72 (<0.1)	12.19,12.41 (0.94)	13.57,13.84 (1.03)	13.78,14.04 (0.29)
5	5.59 (0)	10.52 (0)	10.90,11.01 (<0.1)	13.08,13.32 (1.12)	14.45,14.77 (1.30)	15.53,15.90 (1.54)

Analyte pairs **1 / 4** and **2 / 5**, differing only in their R' groups, displayed different enantio-resolution values. That part of the structure which is believed responsible for inclusion into the CD cavity (R' group) thus played a crucial role, perhaps by holding the molecule in the correct orientation for optimum enantio-recognition.

4.3.4. Complexation with β-CD.

It was not possible to fully investigate the effects of β-CD under the buffer conditions used with the other CDs due to its very low solubility in 30% MeOH (0.64 g/100 mL), Taghvaei and Stewart 1991. Therefore only a 4 mM β-CD buffer was prepared (30% MeOH-KH_2PO_4 50 mM v/v, pH3). No enantio-resolution was observed for any of the analytes at this concentration.

Figure 4.6. Electropherogram for compound **2** in the presence of 35 mM HE-β-CD. FSCE conditions: 30% MeOH - KH_2PO_4 50 mM, pH 2.5. Constant current of 5 μA.



Urea solubilized β-CD (40 mM) was found to enhance chiral separations, with compound resolution values of the order **2>1>5>3>4** (Table 4.6., see p.116). The presence of a *t*-butyl group helped promote resolution with compounds **2>1** and **5>4**. When R' = naphthyl, enantio-resolutions increased with compounds **1>4** and **2>5.** It would seem therefore that under these conditions β-CD favours the recognition of compounds **1** and **2**, the bulkiest and most hydrophobic of the five analytes.

Table 4.6. Resolution values (R_s) and migration times (t_m, mins) for compounds 1-5 in the presence of 40 mM β-CD. FSCE conditions: 30% MeOH - KH_2PO_4 50 mM, pH 2.5, 4M urea. Constant current of 5 μA.

Analyte	Resolution values	Migration time (mins)
1	1.05	12.06, 12.23
2	1.31	10.80, 11.03
3	0.28	10.43, 10.54
4	<0.1	8.76, 8.87
5	0.63	9.86, 10.04

4.3.5. Influence of Methanol (MeOH) on Resolution and Migration Times.

With both HE-β-CD and Ac-β-CD the removal of MeOH had a detrimental effect on resolution. None was observed for any of the analytes with HE-β-CD in the absence of MeOH, whereas they all formerly displayed some resolution when MeOH was present. Ac-β-CD (at 7.4 mM) had only previously resolved compound **2** when MeOH was present. This slight enantio-resolution was removed when MeOH was absent. With Me-β-CD the effect on resolutions seemed more variable. Enantio-resolutions of compounds **1**, **2** and **3** increased when no MeOH was present, whilst those of compounds **4** and **5** decreased. Migration times increased in every case when MeOH was removed.

As stated by Wren and Rowe 1992, organic solvents are believed to change the apparent mobility difference between the analyte enantiomers in FSCE, which in turn can increase or decrease the observed enantioselectivity depending on the CD type and concentration. This finding is mirrored in these results where 30% MeOH was shown to have a beneficial effect on resolution with HE-β-CD and Ac-β-CD, yet it produced both higher and lower resolutions using Me-β-CD, dependent on the analyte structure (and hence its CD complex formation constant). Solvation can radically alter both the geometry and

relative stabilities of various cyclodextrin:analyte conformations (Sherrod 1992), so it is not unexpected that enantio-resolution values and migration times should be affected.

4.3.6. Determination of Complex Formation Constants (K_f).

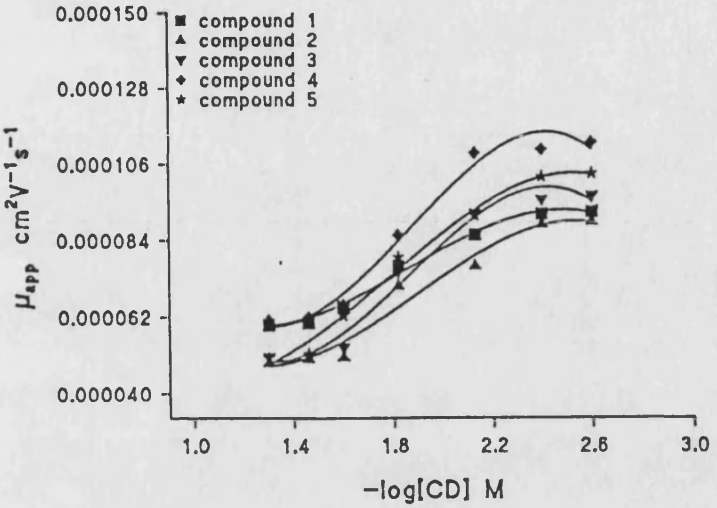
Using the method developed by Gareil *et al.* 1993, plots of μ_{app} versus -log[CD] were constructed for compounds **1-5** with Me-β-CD (Fig. 4.7a) and HE-β-CD (Fig. 4.7b), both on page118. All the plots showed a classical sigmoidal shape from which the K_f values were estimated by measurement of the abscissa of the point of inflection. The K_f values are given in Table 4.7. (see p.118).

The magnitude of K_f values of the compounds is the same with both CDs i.e. **2>1>3>5>4.** It is apparent however that the analytes show stronger binding to the HE-β-CD by virtue of their larger K_f values. HE-β-CD also produced the largest maximum resolution values for compounds **1-4** (compound **5** had an equal maximum R_s value with HE-β-CD and Me-β-CD). Furthermore, the order of binding constants parallels the analyte's hydrophobicity, indicating that this is a major factor in complexation with the CDs.

It was not possible to calculate K_f values with Ac-β-CD as its limited aqueous solubility restricted the number of experimental points that could be plotted, rendering the data inconclusive.

Figure 4.7. μ_{app} versus -log [CD] of compounds 1-5, (a) Me-β-CD and (b) HE-β-CD. FSCE conditions: 30% MeOH - KH_2PO_4 50 mM, pH 2.5. Constant current of 5 μA.

(a)



(b)

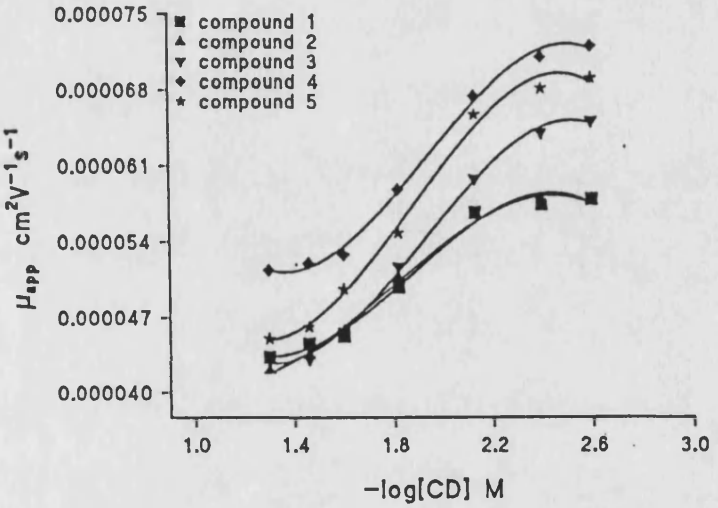


Table 4.7. Estimated K_f (M^{-1}) values of compounds 1-5 with Me-β- and HE-β-CD. FSCE conditions: 30% MeOH - KH_2PO_4 50 mM, pH 2.5. Constant current of 5 μA.

Compound	K_f (M^{-1}) with Me-β-CD	K_f (M^{-1}) with HE-β-CD
1	96	110
2	106	116
3	90	105
4	79	91
5	87	100

4.3.7. Comparison of Results for Propranolol Analogues.

The migration times of compounds **1-5** were longer in the presence of Ac-β-CD than Me-β-CD up to the 15 mM level, suggesting higher association constants. Additionally, these migration times were shorter than those seen with HE-β-CD, suggesting lower association constants. Yet enantio-resolution was much higher for all the analytes with HE-β-CD and also Me-β-CD, except in the case of compound **2**. The strength of binding between the analytes and CDs does not appear to be directly related to enantio-discrimination. The ability of a molecule to form an inclusion complex with a CD does not cause enantio-discrimination *per se* (Fanali 1989), although it is deemed to be a necessary pre-requisite in most instances (Sherrod 1992).

Methylation is known to make the parent β-CD more hydrophobic (Tanaka *et al.* 1985), to increase the cavity depth and enhance the molecular flexibility of the macrocycle due to a reduction in the number of possible hydrogen-bonds between the O(2) and O(3) hydroxyl groups (Harta *et al.* 1984). These changes may explain the analyte enantio-recognition seen with Me-β-CD by allowing a 'better fit' of one enantiomer with the hydrophobic and conformationally flexible Me-β-CD molecule.

HE-β-CD has additional hydrogen-bonding sites on its modified rim, which may account for it displaying the highest maximum resolution values observed in this work and thus allow a closer spatial interaction with the hydrogen-donor/acceptor sites near the asymmetric centre on the analyte alkyl chains. Furthermore, alkylation of a CD (as mentioned above with Me-β-CD) is believed to increase the host macrocycle's flexibility, which can improve solute chiral resolution via an 'induced-fit' interaction (Pálmarsdóttir and Edholm 1994). This could explain the larger K_f values with HE-β-CD than Me-β-CD. The ability of many modified CD's to improve on enantio-recognition values seen with the parent CDs has been described before in other FSCE applications (Fanali 1991, Fanali 1989, Tanaka *et al.* 1985 and Nardi *et al.* 1993).

Perhaps structural changes to the rim of the CD cavity are responsible for the reversal in migration order of compounds **1** and **2** when compared to the results obtained with Ac-β-CD and Me-β-CD. As with Me-β-CD, the existence of a *t*-butyl group on the analytes promoted enantio-recognition in the presence of HE-β-CD, with resolution values of compounds **2>1** and **5>4** at all CD concentrations.

The migration times were faster with β-CD (4 mM) than with the other three CD derivatives, suggesting the possibility of lower CD:analyte formation constants. β-CD did not display analyte separation at 4 mM, unlike HE-β-CD, which was the only CD to resolve compounds **1** and **2** at this CD concentration. Solubilization with urea did allow higher concentrations to be dissolved and produced enantio-recognition in all cases in contrast to Ac-β-CD, where the addition of urea removed all evidence of resolution.

As seen with the CD derivatives, a *t*-butyl group helped promote resolution in the presence of β-CD with compounds **2>1** and **5>4**. When R′ = naphthyl, enantio-resolutions increased with compounds **1>4** and **2>5**, which is directly opposite to the results obtained with Me-β-CD and also in contrast to the resolution order seen for compounds **1** and **4** with HE-β-CD (Matchett *et al.* 1995).

The presence of MeOH was found to have a varied effect on enantio-resolutions which was linked to the findings of Wren and Rowe 1992 who stated that organic solvents altered the apparent mobility difference between enantiomers. Such a change could then alter the resolution values, depending on the type and concentration of CD used.

4.4. CE Investigation of Mequitamium.

From a range of CDs only γ-CD and hydroxypropyl-β-CD (HP-β-CD) had provided any enantio-resolution for mequitamium by HPLC (section 3.4.1.). CE investigations utilised the same group of macrocycles with particular regard to γ-CD, which had proven most successful in the liquid chromatography experiments. The affect of CD type, CD concentration, %MeCN and buffer type/concentration were examined.

4.4.1. Complexation with Various CDs.

The strong positive charge on Mequitamium (see Fig. 3.17.) as a result of its quaternary nitrogen atom, means that it will migrate towards the cathode of a capillary electrophoresis instrument. At low pH's it will not be expected to adhere to the capillary wall due to a pre-existing coverage of positive charge, resulting from buffer ions electrostatically attracted to ionised silanol groups on the surface. There is thus no 'stationary phase' interaction, unlike that observed in HPLC experiments (section 3.4.).

Table 4.8. (see p.122) shows the migration times and enantio-separations of mequitamium in the presence of various CD molecules, three of which demonstrated chiral resolution for the analyte. In all cases where separation occured, repeated injections with the individual enantiomers showed that the R-isomer was migrating first i.e. its degree of complexation with the CD was lower than that of the S-isomer. All migration times and resolutions are a mean of two separate injections.

Table 4.8. Migration times and Resolution values for mequitamium with various CDs. FSCE conditions: 9.5% MeCN-NaH_2Citrate 50 mM, pH 3.5, containing 10 mM CD. Bare silica capillary (50 cm x 50 μm), constant voltage 15.1 KV (i ≈ 20 μA). Gravity injection 15 sec., height 90 mm. Sample concentration 0.2 mg/ml, dissolved in running buffer.

Type of cyclodextrin	Migration time (mins)	Resolution value
none	15.54	-
α	15.96	0.0
β	19.32	0.0
γ	21.84, 22.24	1.72
HP-α	16.34	0.0
HP-β	24.74, 25.02	1.22
HP-γ	22.00, 22.20	0.86
Me-β	17.01	0.0
HE-β	20.24	0.0

Modification of the parent γ-CD hydroxyl groups to form the hydroxypropyl derivative resulted in a lowering of enantioselectivity although the analyte migration times did not alter appreciably. HP-β-CD proved a better chiral discriminating agent than HP-γ-CD, however the parent β-CD molecule was unable to resolve the mequitamium enantiomers. Electropherograms of mequitamium showing clear enantio-resolution in the presence of the different CDs are shown in Figure 4.8. (see p.123). Derivatization with hydroxypropyl chains thus proved beneficial for β-CD but detrimental for γ-CD. α-CD and HP-α-CD had no effect on resolution and little effect on migration times, as did Me and HE-β-CD.

Optical resolution in aqueous systems depends on the analyte forming a close spatial fit with the cavity of the host CD molecule and thence a chiral interaction via the fixed hydroxyls on the CD rim. Derivatization (e.g. of γ-CD to form the hydroxypropyl

macrocycle) affects the hosts hydrogen bonding ability and may also alter the size (depth and width) of its cavity (Meier-Augenstein *et al.* 1992 and Pálmarsdóttir and Edholm 1994). Such changes could account for the reduction in enantio-selectivity seen with HP-γ-CD relative to γ-CD.

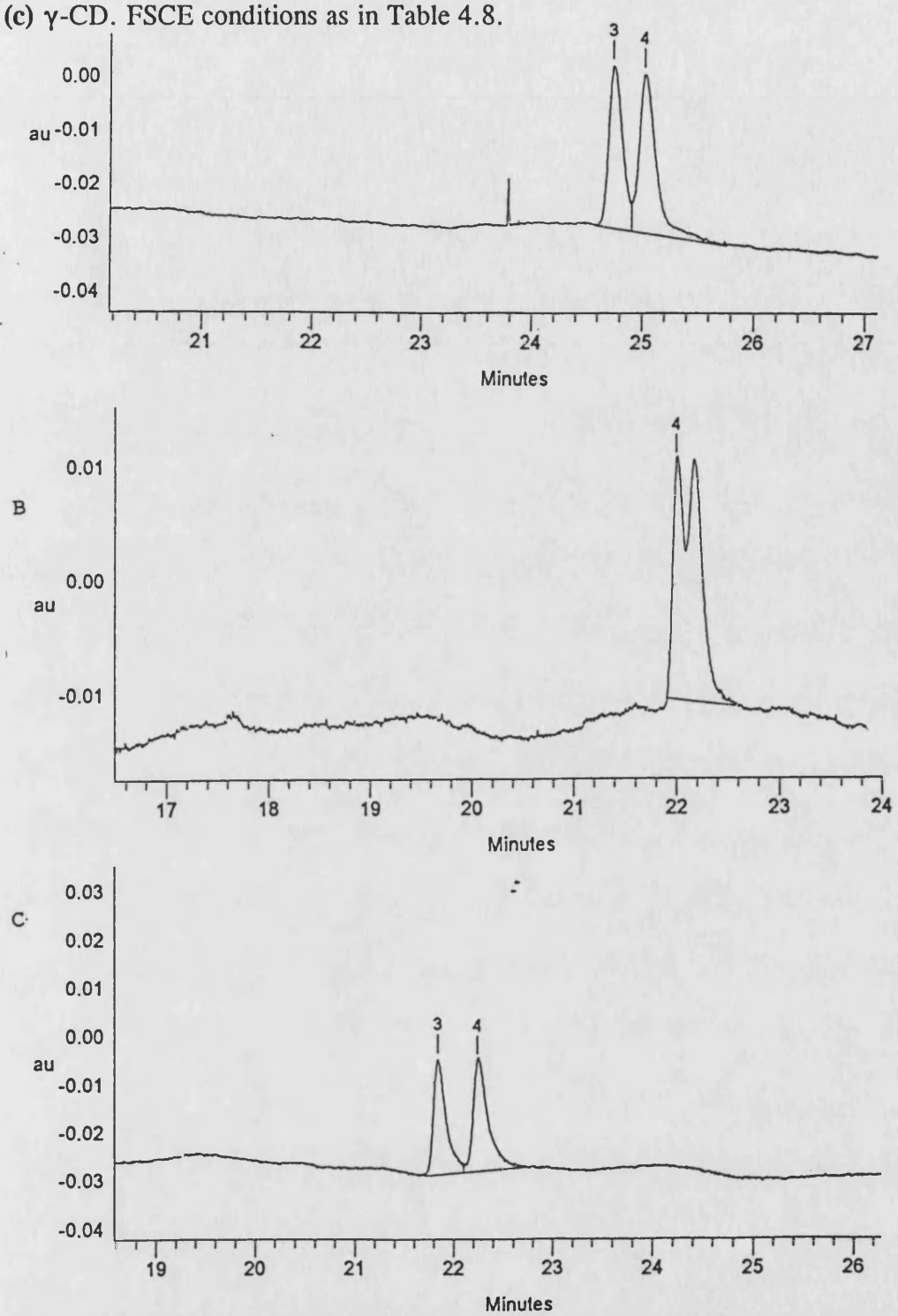
Conversely, an increase in cavity size and/or extension of the hydrogen bonding of the smaller β-CD molecule (to form HP-β-CD) may have allowed a more favourable interaction with the large mequitamium structure, rationalising the observed improvement in chiral discrimination. However HE-β-CD and Me-β-CD did not resolve the mequitamium enantiomers, suggesting that the length of the alkyl chains on the CD hydroxyl groups was a major factor in the chiral recognition process.

The small size of the α-CD cavity, 0.5 - 0.6 nm, (Technicol 1992) seems the most likely reason for its lack of enantio-resolution of mequitamium and the insignificant change in migration time of the free analyte upon the addition of α-CD to the FSCE buffer. If the host and guest molecules cannot form an inclusion complex in aqueous solution because of their steric incompatibility, then no optical resolution will occur. HP-α-CD caused the analyte migration time to increase only slightly (< 1 minute) and produced no resolution. The size of the host cavity is clearly important for complexation as is the overall derivatization of the rim hydroxyl groups.

4.4.2. Effect of Operating Parameters on Resolution and Migration Times.

It was not possible to observe chiral discrimination when using KH_2PO_4 as the buffer component even in the presence of γ-CD. Upon switching to NaH_2citrate however, chiral resolution was clearly evident. Bechet *et al.* 1994, found that different buffers had varied

Figure 4.8. Electropherograms of mequitamium, **(a)** with HP-β -CD, **(b)** HP-γ-CD and **(c)** γ-CD. FSCE conditions as in Table 4.8.



effects on analyte enantio-resolutions and migration times in FSCE, which they attributed to their ability to modulate electroendosmotic flow. It is also known that buffer molecules

compete with analytes for penetration into CD cavities (Technicol 1992). Altria *et al.* 1992 suggested the formation of an ion-pair between certain buffer ions and the CD:analyte complex, to account for optimum chiral resolutions observed under certain buffer conditions. The nature of the buffer, which is often selected capriciously, clearly influenced the resulting analysis.

Increasing the buffer concentration will reduce the electroendosmotic flow (Weinberger 1991) and so may lead to an increase in analyte migration times. However it will also affect the complexation equilibria by enhancing the displacement of analytes from the CD cavity, which means the analyte will spend longer in the faster moving uncomplexed form. Upon using a 75 mM or 100 mM NaH_2citrate buffer (as opposed to a 50 mM buffer), migration times decreased and chiral resolution was negated, which suggests that here, the aforementioned equilibria shift predominates.

The level of organic modifier (MeCN) present in the running buffer had a strong affect on migration times and enantio-resolution values. Using a fixed γ-CD concentration of 10 mM, the % of MeCN was varied between 5 and 19.5% (at least 5% MeCN was necessary to ensure dissolution of the analyte). The migration time and chiral resolution values initially increased at 9.5% MeCN and then both successively decreased as the MeCN concentration was raised further (Table 4.9., see p.126).

A CD containing buffer with a %MeCN concentration of 9.5% displayed the highest resolution value and longest migration time of the mequitamium enantiomers, suggesting a longer analyte:CD interaction allows a better chiral discrimination interaction to occur. Increasing the %MeCN above this optimum level led to a decrease in migration times (i.e. less CD:analyte interaction) and a concurrent fall in chiral resolution as a result of

Table 4.9. Affect on migration time (mins) and resolution value of MeCN addition. FSCE conditions: $NaH_2Citrate$ 50 mM, pH 3.5, 10 mM γ-CD, containing MeCN. Bare silica capillary (50 cm x 50 μm), constant voltage 15.1 KV (i≈ 20 μA). Gravity injection 15 sec., height 90 mm. Mequitamium concentration 0.2 mg/ml, dissolved in running buffer.

MeCN %	5%	9.5%	12%	15%	19.5%
Retention time (mins)	12.08, 12.16	16.22, 16.60	12.34, 12.50	10.36, 10.42	9.88, 9.98
Resolution	0.24	1.53	0.63	0.21	<0.1

the increasing numbers of MeCN molecules displacing mequitamium from the γ-CD cavity (see Fig. 4.9., p.127). Furthermore, MeCN has been shown to increase electroendosmotic flow (Fujiwara and Honda 1987), which may also have contributed to the noted quickening of analyte migration times.

4.4.3. Influence of γ-CD.

The most enantio-selective CD additive for the mequitamium enantiomers was seen to be γ-CD (Table 4.8., see p.122). It is well known that the concentration of chiral selector can affect enantiomeric optical resolutions and migration times (Penn *et al.* 1993, Snopek *et al.* 1991 and Nardi *et al.* 1992). If the two enantiomers have different affinities for the CD i.e. different Kf values, and the electrophoretic mobilities of the free and complexed forms are different, then chiral resolution may be observed (Wren and Rowe 1992). Consequently the concentration of γ-CD was varied between 0.0 and 50 mM and the resultant migration times, electrophoretic mobilities (μ_{app}) and chiral resolutions of mequitamium are given in Table 4.10. (see p.128).

Figure 4.9. Pherograms of mequitamium in presence of increasing MeCN content. FSCE conditions: as in Table 4.7., plus (a) 5%MeCN, (b) 9.5%MeCN, (c) 12%MeCN, (d) 15%MeCN and (e) 19.5%MeCN.

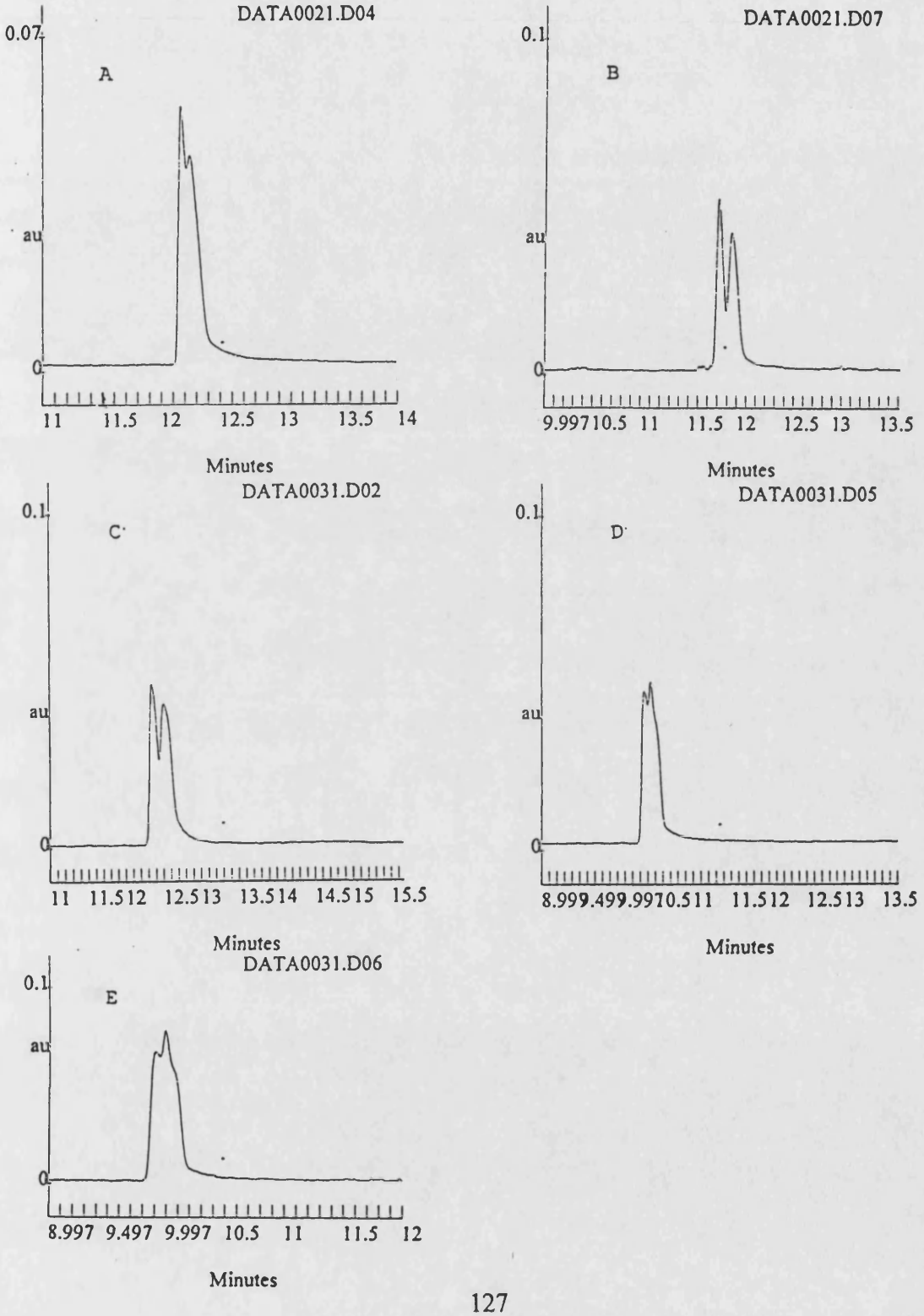


Table 4.10. Effect of γ-CD concentration on FSCE examination of mequitamium.

FSCE conditions: as Table 4.9., with varying γ-CD concentration.

γ-CD mM	migration time (mins)	resolution value	-log [γ-CD] mM	μ_{app} x10^{-5} $cm_2V_{-1}S_{-1}$ peak one	μ_{app} x10^{-5} $cm_2V_{-1}S_{-1}$ peak two	$\Delta\mu_{app}$ x10^{-5} $cm^2V^{-1}s^{-1}$
0	12.08	0	-	20.44	-	-
0.15	12.90	0	3.82	19.10	-	-
0.5	13.15, 13.23	0.22	3.30	18.78	18.67	0.11
1.0	14.22, 14.32	0.63	3.00	17.36	17.25	0.11
2.0	15.14, 15.32	1.11	2.70	16.31	16.12	0.19
2.5	17.26, 17.54	1.78	2.60	14.31	14.08	0.23
5.0	20.72, 21.12	1.82	2.30	11.92	11.69	0.23
10	22.22, 22.60	1.72	2.00	11.11	10.93	0.18
15	24.44, 24.80	1.41	1.82	10.10	9.96	0.14
20	26.44, 26.80	1.30	1.70	9.34	9.22	0.12
30	30.28, 30.62	1.02	1.52	8.15	8.07	0.08
50	30.64	0	1.30	8.06	-	-

Increasing the CD concentration over the entire range caused the enantiomer migration times to successively lengthen, indicating stronger CD:analyte complexation. However it is clear that this did not always lead to a concurrent improvement in optical resolution. The maximum resolution value occurred at a γ-CD level of 5 mM, whilst higher concentrations only produced lower resolutions and longer migration times. The enantiomer mobility difference, $\Delta\mu_{app}$, plateaued between 2.5 and 5.0 mM γ-CD. Equation 4.11. (section 4.1.) explains these phenomena, whereby the optimum enantiomer mobility difference will occur between some zero and infinite CD concentration as will the maxiumum resolution. However both values are not necessarily identical as demonstrated by Penn *et al.* 1993 (see also equations 4.12 and 4.13). This is in agreement with the results found here, with maximum resolution observed at 5 mM γ-CD and maximum $\Delta\mu_{app}$ seen to range from 2.5 to 5.0 mM γ-CD. Figure 4.10. (see p.130) shows representative electropherograms of mequitamium as the γ-CD concentration was continually increased.

4.4.4. Determination of Complex Association Constants (K_f).

Using the method described by Gariel *et al.* 1993 for FSCE experiments (applied in sections 4.2.4. and 4.3.6.), the complex association constants for the enantiomers of mequitamium, denoted K_S and K_R, in the presence of γ-CD where found by plotting μ_{app} *versus* -log[CD] M (Fig. 4.11., see p.131). These values were estimated to be 355 M^{-1} and 316 M^{-1} respectively. The optimum CD concentration for mobility difference, μ_{app}, which can be calculated from equation 4.12., was then determined to be 3.0 mM γ-CD. Examination of Table 4.10. shows that this value lies within the plateau region observed for μ_{app} i.e. 2.5 to 5.0 mM and so is in good agreement with these results.

Figure 4.10. Electropherograms of mequitamium with increasing γ-CD concentration. FSCE conditions as Table 4.9.

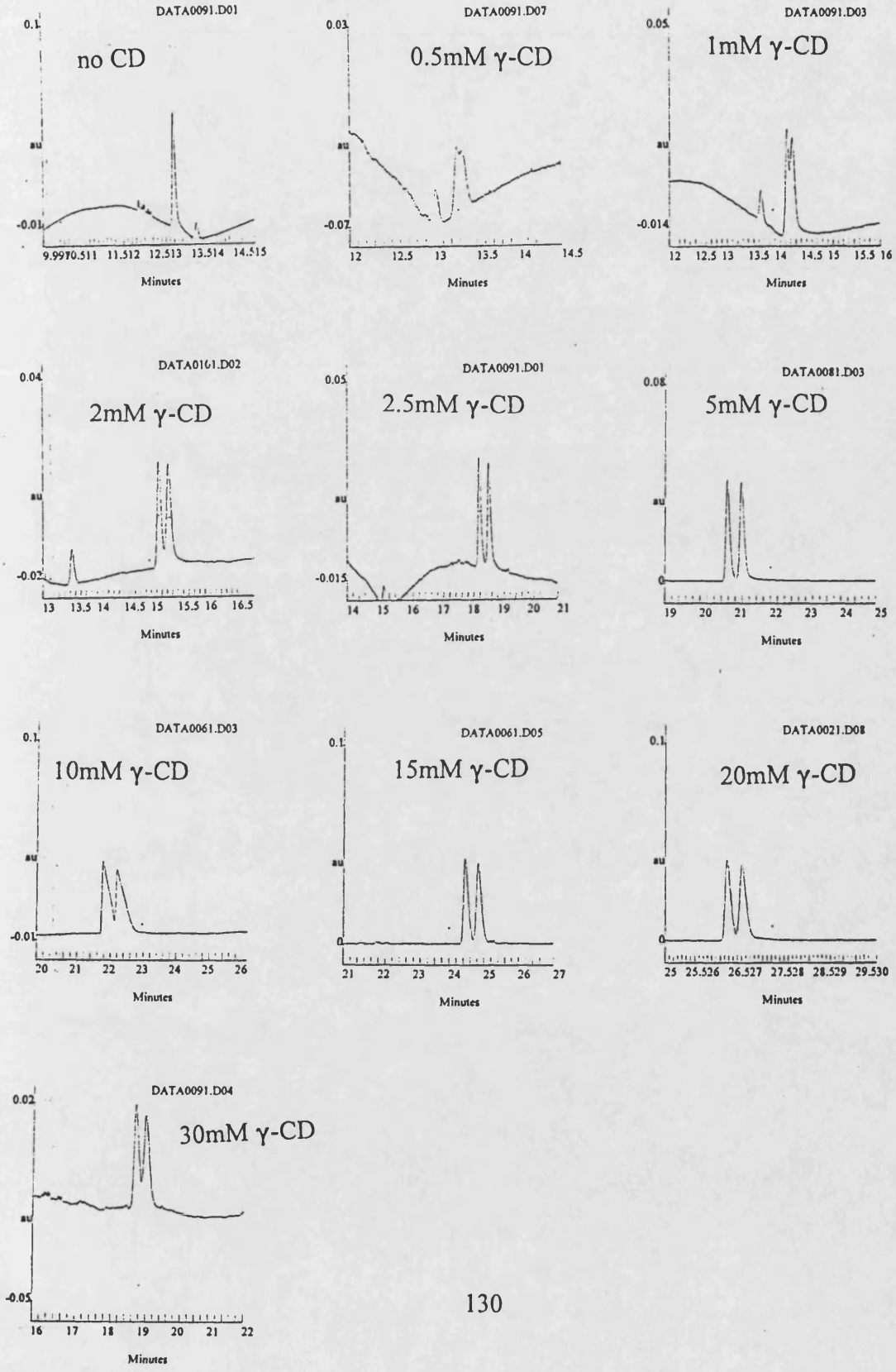
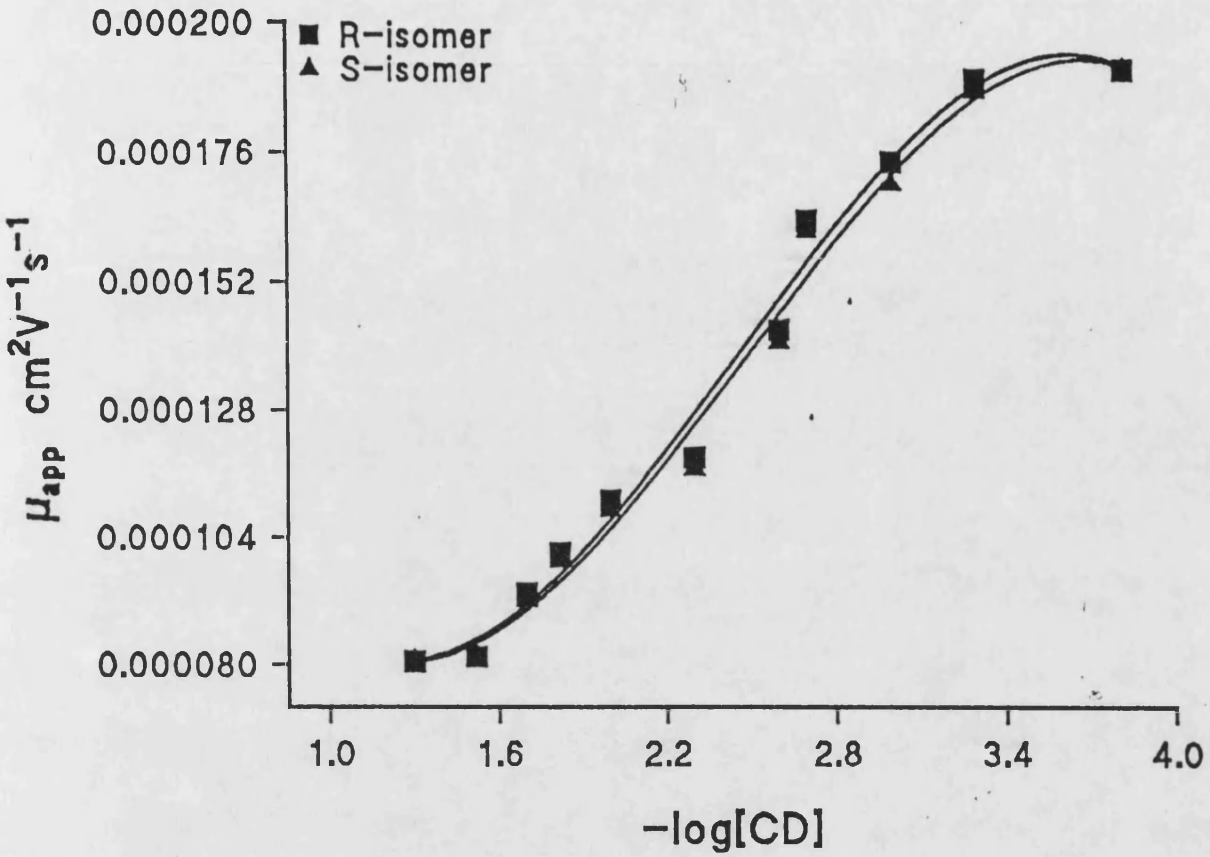


Fig. 4.11. μ_{app} **versus -log [CD] for mequitamium with γ-CD.** FSCE conditions as in Table 4.9.



Furthermore, the concentration of CD required for optimum enantio-resolution can be found using equation 4.13. The mobility of the γ-CD:mequitamium complex, μ_{-} , was taken as the mobility value in the presence of 50 mM γ-CD i.e. that part of the curve in Fig. 4.11. which corresponds to the analyte being fully complex. μ_{eo}, the electroendosmotic flow rate, was estimated using MeCN as the neutral marker and found to be 3.8 x10 $^{-6}$ cm^2 V^{-1} s^1 . The electrophoretic mobility of mequitamium in the absence of γ-CD was 20.44 x10 $^{-5}$ cm^2 V^{-1} s^1 . Substitution of these values into equation 4.13. indicated that a γ-CD concentration of 4.7 mM would have provided optimum enantio-resolution. Table 4.8. shows that 5 mM γ-CD gave the highest observed resolution and is therefore in good agreement with the predictive theory.

CHAPTER FIVE

NMR RESULTS AND DISCUSSION

5.1. Introduction.

NMR can be effectively used to assess the interaction sites between CDs and various chiral guest molecules. Chemical shifts, δ's, and coupling constants, *J*, of both host and guest nuclei are sensitive to changes that have occurred within the molecular environment of the complex. Hence NMR can provide an insight into the molecular positions and orientations adopted during the interaction process.

5.1.1. ^{1}H-NMR Spectra of CDs.

Table 5.1. gives the typical ^{1}H-NMR signals generated by the three parent CDs, α-, β- and γ-CD when dissolved in D_2O (from Casy and Mercer 1988). Figures 5.1. (a) and (b) show respectively, the proton numbering pattern assigned to each glucose residue in the parent CDs and the overall shape of the CD macrocycle when the residues are linked together.

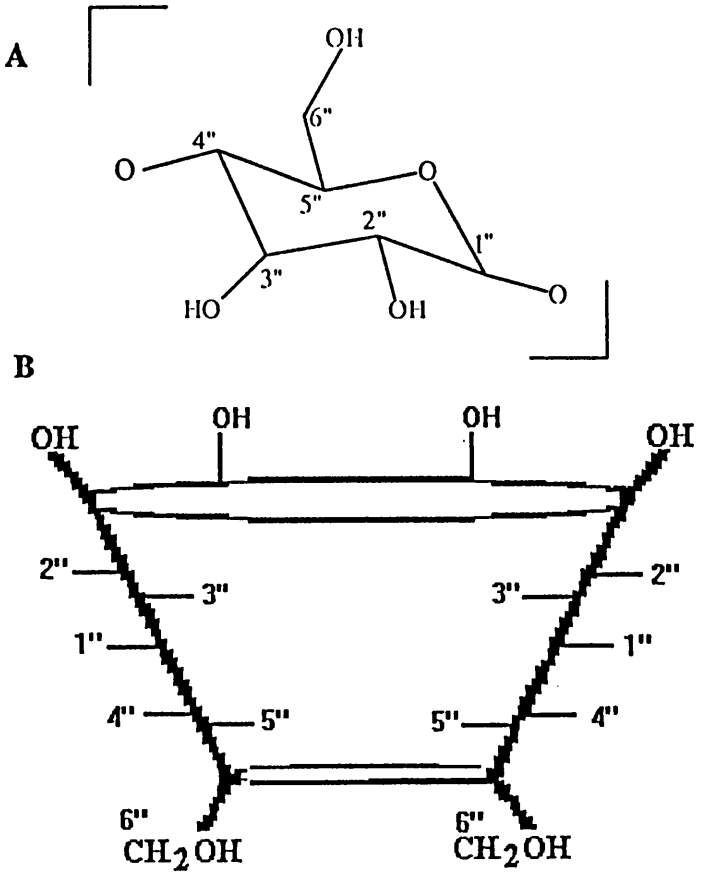
Table 5.1. ^{1}H-NMR signals and multiplet separations (Hz) in parentheses of α, β and γ-CD. (at 400 MHz).

Cyclodextrin	H-1	H-2	H-3	H-4	H-5	6-CH_2
α-CD	5.03 d (3.5)	3.61 dd (3.4, 10)	3.96 t (9.5)	3.59 t (9)	a	a
β-CD	5.05 d (4)	3.62 dd (3.5, 9.8)	3.94 t (9.5)	3.56 bt (9)	a	a
γ-CD	5.08 d (3.7)	3.63 dd (3.4, 9.8)	3.91 t (9.5)	3.59 t (9.5)	a	a

[a] Overlap to form multiplet centered near 3.85 ppm.

The proton spectrum of free β-CD in D_2O was initially assigned by Demarco and Thakkar 1970. Wood *et al.* 1977 (and ref. 21 cited therein) established that each glucose residue in the parent CDs exists in a similar chemical environment to that of the others, so that the overall NMR spectrum of the macrocycle does not differ greatly from that of an individual residue. Many other workers have gone on to assign the spectra of various CD derivatives e.g. methylated β-CDs using ^{13}C-NMR (Szejtli *et al.* 1980), methylated α and β-CDs using ^{1}H and ^{13}C-NMR (Yamamoto *et al.* 1987 and Inoue *et al.* 1986), alkylated β-CDs using ^{1}H and ^{13}C-NMR 1D and 2D experiments (Meier-Augenstein *et al.* 1992 and Hirayama *et al.* 1993) and peracetylated and methylated β-CDs (Tezuka and Hermawan 1994). Takahashi 1993 synthesised a mono-tyrosylamino derivatised CD and reported that the glucopyranose units were magnetically non-equivalent when examined by ^{13}C-NMR.

Figure 5.1. (a) and (b). CD proton numbering scheme and its overall structure.



CDs are soluble in water and D_2O so they can be applied directly to water soluble chiral analytes e.g. many therapeutic compounds are in the form of protonated salts or alkali metal salts of acids. Thus, they can be easily used for the examination of a wide range of pharmaceutical compounds. CDs also have no broadening effect on ^{1}H-NMR signals and their δ range is relatively narrow (3.5 - 5.1 ppm) so they do not greatly obscure the spectra of many compounds of interest (Casy 1993a).

Casu *et al.* 1968 were among the first to show how the glucose residues adopt the $^4C_1$ chair conformation in solution, using NMR analysis. X-ray diffraction studies (e.g. Lindner and Saenger 1982) have since proved this to be the case. Intramolecular hydrogen bonding is known to occur in solution between the secondary hydroxyl groups of underivatised CDs (St-Jacques *et al.* 1976 and Onda *et al.* 1988). When dissolved in DMSO (and also in D_2O), the OH group in position (2) and that in position (3), are seen to form a hydrogen bond where OH(3) is the main hydrogen donor. This bond helps to maintain the structure of the CDs and may explain why some derivatised CDs possess a more distorted / flexible structure when the hydrogen bonding capacity at these points has been lessened (Inoue *et al.* 1987, Ventura *et al.* 1994 and Myles *et al.* 1994).

5.1.2. Guest Induced Chemical Shifts of CDs.

Upon complexation with many guest molecules, CDs display characteristic changes in the chemical shifts of their protons. Aromatic guest species in particular tend to cause the inner H3" and H5" CD protons to be shifted upfield e.g. up to -0.25 ppm shifts (Smith *et al.* 1994) and -0.288 shifts (Yoshida *et al.* 1990a) have been reported i.e. the protons are shielded by the diamagnetic anisotropy of the penetrating aromatic group. The two H6" protons can also experience relatively large shifts, which may be attributable to their proximity to the CD cavity. These phenomena have been widely

reported by other workers e.g. Komiyama and Hirai 1980a, Nakajima *et al.* 1984, Yamashoji *et al.* 1990, Rekharsky *et al.* 1994, Azaroual-Bellanger and Perly 1994.

The resonances of the other CD protons, H1", H2", H4" and H6a" / H6b", generally do not show $\Delta\delta$ changes as large as those of the inner H3" and H5" protons. Bergeron *et al.* 1978 noted this effect between α-CD and benzoic acid, as did Smith *et al.* 1994 (when examining the interaction of catechin with α-, β- and γ-CD); Mulinacci *et al.* 1993 found H3" β-CD signals shifted by more than four times the extent of H1" and H2" signals in the presence of ibuproxam; Jaime *et al.* 1990 noted a continuous shielding of H3" and H5" protons when the ratio between β-CD and 1-bromoadamantane was varied but that no changes occurred for the outer β-CD protons, whilst Uccello-Barretta *et al.* 1993 noted only minor shifts for H1", H2" and H4" protons of β-CD when complexing with (S)-(+)-fenoprofen. This evidence reinforces the view that inclusion complexation is the primary mechanism by which CDs interact with guest molecules in aqueous environments and that those protons located on the 'outer-face' of the macrocycle (H1" and H4") do not generally come into intimate contact with the included species.

5.1.3. NMR Equilibria for Complex Formation.

The association constant, K_f, can be evaluated from NMR measurements using graphical procedures if the chemical shift of one species is observed in the presence of a large excess of another species. Assuming that a 1:1 complex, A.CD, is formed between a guest molecule, A, and a cyclodextrin, CD, then the following equations are applicable;

$$[A] + [CD] \rightleftharpoons [A.CD] \qquad \text{(Equation 5.01.)}$$

$$K_f \text{ (association constant)} = [A.CD] / [A] + [CD] \qquad \text{(Equation 5.02.)}$$

$$[A.CD] = \Delta\delta_{obs} \cdot [A]_t \cdot 1/\Delta\delta_c \qquad \text{(Equation 5.03.)}$$

where $[A]_t$ is the total guest concentration and $\Delta\delta_{obs}$ and $\Delta\delta_c$ are given by,

$$\Delta\delta_{obs} = \delta_{obs} - \delta_f \qquad \text{(Equation 5.04.)}$$

$$\Delta\delta_c = \delta_{A.CD} - \delta_f \qquad \text{(Equation 5.05.)}$$

with δ_f and $\delta_{A.CD}$ representing the chemical shifts of a given nuclei in the free and complexed forms respectively. It may be shown that the measured chemical shift is related to the total CD concentration, $[CD]_t$, by,

$$\Delta\delta_{obs} = K_f \cdot [CD]_t \cdot \Delta\delta_c \cdot (1 + K_f \cdot [CD]_t)^{-1} \qquad \text{(Equation 5.06.)}$$

The stability of analyte-CD complexes can then be measured via equation 5.06. by varying the CD concentration and observing its effect on the chemical shifts of specific nuclei. Bergeron *et al.* 1977 provided much of the mathematical basis for the above treatment, using a modified form of the Hildebrand-Benesi equation (see Benesi and Hildebrand 1949 ref.12 cited in Bergeron *et al.* 1977), to calculate the dissociation constants between α-CD and a series of phenol derivatives. By plotting $1/\Delta\delta_{obs}$ *versus* 1/[CD] a linear relationship results, where the slope is $1/(K_f \cdot \Delta\delta_c)$, the x-axis intercept is $-K_f$ and the y-axis intercept is $1/\Delta\delta_c$. Various workers have since applied the same principles to other CDs and guest compounds e.g. Smith *et al.* 1989, Wenz and von der Bey 1988 and Casy *et al.* 1991.

Rearranging equation 5.06. to,

$$\Delta\delta_{obs} \cdot ([CD]_t)^{-1} = K_f \cdot (\Delta\delta_c - \Delta\delta_{obs}) \qquad \text{(Equation 5.07.)}$$

allows the association constant, K_f, to be evaluated by plotting $\Delta\delta_{obs}$/[CD] *versus* $\Delta\delta_{obs}$ where the slope equals $-K_f$. This is the Foster-Fyfe method (Foster and Fyfe 1965), which enables a more straightforward graphical determination of K_f than the Hildebrand-Benesi method. It too has been used by other workers e.g. Uccello-Barretta *et al.* 1993.

If the association constants for enantiomers with a given CD are very similar, the observed enantiotrophic shifts may be attributed to the different orientation of the enantiomers in the CD cavity (Uekama *et al.* 1985).

5.1.4. 2D NMR Techniques - NOESY and ROESY.

NOESY (Nuclear Overhauser Effect Spectroscopy) allows the spatial visualization of 3D interactions between e.g. a CD and guest molecule, whose nuclei are close enough together in space to affect each other through dipole-dipole interaction mechanisms. The magnitude of the NOE depends on the distance between interacting nuclei and diminshes dramatically (to the sixth power) as the distance between saturated and observed nuclei increases (Field 1989). Bekers *et al.* 1991 were able to partially determine the mode of interaction between γ-CD and doxorubicin using NOESY spectra but could not unequivocally assign the spectra due to extensive peak overlap. Bates *et al.* 1994 tried to observe NOE's between the protons of 'poly'-O-octyl-α-CD and the aryl protons of ephedrinium ion but they were also unsucessful. Hartzell *et al.* 1993 used proton 2D exchange spectroscopy (EXSY), which employs a phase sensitive NOESY pulse sequence, to determine rate constants for the exchange process between β-CD and TCNQ- (tetracyanoquinodimethane), corroborating other evidence that they exist as a bonded dimer in solution.

The ROESY (Rotating Frame Nuclear Overhauser Effect Spectroscopy) or CAMELSPIN experiment is a variation of the NOESY technique, where a spin-locking field is used to

view the NOE's within a rotating frame of reference. The magnitude of the NOE depends, amongst other factors, on the molecular tumbling rate (correlation time) of the molecule. Small molecules tumble rapidly in solution giving positive NOE's, whilst the opposite is true for larger molecules. ROESY allows extremely small NOE's, which can result from the unfavorable correlation times of host-guest complexes with a molecular weight of around 1000 - 2000, to be observed at high field strengths. This technique was first described by Bothner-By *et al.* 1984 and causes all NOE's to appear positive under any value of $\omega\tau_c$, where ω is the angular Larmor frequency and τ_c is the motional correlation time of the complex (Neuhaus and Williamson 1989). Many CD complexes have a molecular weight within the region where NOE's are difficult to detect with NOESY experiments, so lending themselves to investigation by the ROESY technique.

Schneider *et al.* 1991 used 2D ROESY experiments to prove greater inclusion of phenyl and naphthyl moieties by γ-CD than β- and α-CD. Amato *et al.* 1992 found that ROESY evidence of dipolar contacts between β-CD and a chiral anti-amnesic drug supported molecular modelling results of the complex's geometry. Amato *et al.* 1993 were further able to show via ROESY experiments that β-CD more fully complexed a selected chiral cognition activator drug than did α-CD, which was also shown to be unable to chirally discriminate between the drug enantiomers as opposed to β-CD. Ndou *et al.* 1993 used 2D ROESY to show the preferential location of ephedrine inside β-CD with the guest alkyl chain in close proximity to the bulk aqueous environment on the primary CD side and the aromatic group residing in the β-CD cavity.

5.1.5. Determination of Complex Stoichiometry.

It is posible to determine the host-guest complexation ratio (stoichiometry) when exchange is rapid on the NMR time scale, by recording the spectra of solutions where the ratio of the two constituents is varied but the overall molar concentration of the

solution remains constant. If a physical parameter which is related to the concentration of the complex e.g. proton chemical shift can be measured and plotted as a function of the A:CD ratio, a maximum value will be reached which shows the stoichiometry of the complex. This is known as a Job plot (Job 1928) and may be visualised by plotting δ_{obs} Hz·[CD]/[CD]+[A] *versus* [CD]/[CD]+[A] where δ_{obs} Hz is the δ value in Hertz.

Greatbanks and Pickford 1987 performed a Job plot with β-CD and propranolol HCl in D_2O at 30°C and concluded that they complexed in a 1:1 ratio. Bekers *et al.* 1991 calculated the stoichiometric ratios between two anthracyclines and γ-CD. They found that a 1:1 ratio was formed for both guest molecules at a low pH of three, which was necessary to allow maximum stability for the guest molecules. Fronza *et al.* 1992 also estimated a 1:1 host-guest complexation ratio between β-CD and piroxicam, a nonsteroidal anti-inflammatory. They were investigating means of improving the absorption of the drug from the gastrointestinal tract by preparing it in a carrier molecule (β-CD). Djedaïni *et al.* 1990 calculated a 1:1 complex ratio between β-CD and indomethacin in aqueous solution at p^2H, in order to mimic physiological conditions.

5.1.6. CDs in ^1H and ^{13}C-NMR Spectroscopy.

If equilibrium between analyte-CD complexes and the free analytes is achieved rapidly on the NMR time scale, the NMR signals observed will reflect the net resultant of contributions of the free and bound forms (Casy 1993a). Duplication of certain guest signals in the presence of CDs is indicative of the formation of diastereomeric complexes (Casy and Mercer 1988) and may arise as a result of differences in complex stabilities and / or intrinsic chemical shifts (Cooper *et al.* 1991). There has been a great deal of attention paid to the effects of CDs on the NMR spectra of included species. In examples of early work, Bergeron and Rowan 1976 provided evidence (via Δδ's) for the formation of inclusion complexes between α- and β-CD with sodium *p*-nitrophenolate using ^1H-

NMR. Wood *et al.* 1977 examined the interaction of α-CD in D_2O with *p*-iodoaniline and suggested that the formation of an inclusion complex was responsible for the observed Δδ changes. Uekama *et al.* 1977 measured ^{13}C spin-lattice relaxation times (T_1) of β-CD and sulfathiazole (a sulphur containing drug) and suggested that the overall decrease in T_1 was the result of inclusion complex formation, whilst Komiyama and Hirai 1980b also used ^{13}C-NMR to elucidate the conformation of α-CD and 3-nitrophenol in solution.

CDs have been used in NMR to observe complexation with fullerenes (Andersson *et al.* 1994), atropisomers (Casy 1993b), pharmaceuticals (El-Gendy and El-Gendy 1993 and Hanna and Lau-Cam 1993), glycolipids (Casu *et al.* 1990) and cyclohexane derivatives (Rekharsky *et al.* 1994). Inoue *et al.* 1985 demonstrated for the first time how the orientation of *p*-nitrophenol with methyl-α-CD in solution differed from that seen in the solid state by examining ^1H Δδ's of the included guest. Xiang and Anderson 1990 used ^1H-NMR Δδ's of host and guest compounds to help show the complexation structure between HP-β-CD and several purine nucleosides was enhanced when the guest molecules were present in an ionically neutral form. Suzuki *et al.* 1990 showed how azo dyes protruded from the narrow rim of various CDs by virtue of induced shifts of both the CD CH_2OH groups and of certain nuclei of the guest species. Inoue *et al.* 1987 were able to determine the mode of inclusion between various CDs and phenylalanine by considering Δδ's of host and guest nuclei. Coleman *et al.* 1988 confirmed molecular modelling assignments for menthol and β-CD using ^1H-NMR, as did Weise *et al.* 1989 with β-CD and indomethacin, whilst Lehmann *et al.* 1991 probed the formation of inclusion complexes with permethylated and peracetylated β-CDs and a variety of aromatic guests in D_2O and CD_3OD, observing complexation in D_2O only.

Molecular complexes of various explosives with α-, β- and γ-CD have been studied by Cahill and Bulusu 1993 using ^1H spin-lattice relaxation times (T1) to show which explosives bind more strongly in solution with a view to improving the long term stability of the explosive materials. Wenzel *et al.* 1994 and Sherry *et al.* 1994 examined

lanthanide ion induced chemical shifts of CDs and selected hosts e.g. propranolol, ibuprofen and pheniramine. They found that lanthanide ions such as dysprosium were able to enhance the the extent of chiral resolution seen in the NMR spectra for the enantiomers. Pirkle and Sikkenga 1975, were the first to demonstrate this effect.

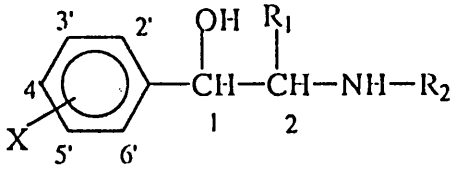
5.1.7. CDs in Multinuclear NMR Spectroscopy.

Analytes bearing nuclei other than ^{1}H and ^{13}C, which are sensitive to NMR spectroscopy, have been examined using a variety of CDs. MacNicol and Rycroft 1977 were able to induce enantiomeric shifts of fluorine bearing analytes (using ^{19}F-NMR) in the presence of α and β-CD, which they attributed to guest binding inside the CD cavities. Dyllick-Brenzinger and Roberts 1980 measured ^{15}N chemical shift changes of a racemic tetrahydroquinoline in the presence of β-CD and found that the nature of the solvent was very important when quantifying the shift values. Taylor *et al.* 1991 used ^{19}F-NMR to examine the signal splitting occuring with Me-β-CD and a proprietary compound bearing a trifluoromethyl group. They found the spectrum was greatly simplified compared to ^{1}H-NMR and that several other derivatised CDs were also able to induce partial signal splitting of the fluorine containing moiety. Line broadening of ^{81}Br-NMR signals caused by derivatised α-CDs has been noted by Yamashoji *et al.* 1993.

5.2. NMR Investigation of Phenethylamines.

Figure 5.2. shows the structures of those phenethylamines examined and the numbering scheme adopted for NMR assignments.

Figure 5.2. Numbering scheme used in NMR assignment of phenethylamines.



Compound	name	X	R_1	R_2
1	ephedrine	H	CH_3	CH_3
2	oxedrine	4-OH	H	CH_3
3	oxilofrine (oxyephedrine)	4-OH	CH_3	CH_3
4	norfenefrine	3-OH	H	H
5	etilefrine	3-OH	H	CH_2CH_3
6	orciprenaline	3,5-diOH	H	$CH(CH_3)_2$
7	noradrenaline (norepinephrine)	3,4-diOH	H	H
8	isoprenaline	3,4-diOH	H	$CH(CH_3)_2$
9	salbutamol	3-CH_2OH,4-OH	H	$C(CH_3)_3$

It had already been demonstrated that β- and Ac-β-CD were able to chirally resolve members of this group of analytes by CE (section 4.2.) and that peracetyl-β-CD could separate the enantiomers of three phenethylamines by HPLC (section 3.2.4.). Unfortunately an investigation by NMR of the 'host-guest' interactions seen in the HPLC experiments was inconclusive. This occurred because it was necessary to use a deuterated

organic solvent ($CDCl_3$) to allow the dissolution of sufficient quantities of peracetyl-β-CD for NMR analysis. However this very non-polar environment understandably did not favour inclusion complex formation, resulting in the peracetyl-β-CD:analyte signals showing little or no chemical shift changes (0.002 Δδppm) compared to those in the free state (Lehmann *et al.* 1991 noted a similar problem when studying peraceyl-β-CD by NMR). The possibilty that peracetyl-β-CD would be able to interact with the solutes via a 'polar-organic' mechanism (section 3.1.3.), may have been precluded by the total absence of hydrogen donor groups in the 2, 3 and 6 positions.

5.2.1. Analyte Complexation with β and Ac-β-CD.

The successful CE buffer conditions were replicated for the NMR measurements, using the nine phenethylamines as 'guest' molecules, with β and Ac-β-CD as the 'hosts'. For the measurement of complex-induced shifts, sufficient quantities of the analytes with and without the appropriate CD were dissolved in deuterated 0.1M phosphate buffer equivalent to pH 4.5 to give 12 mmol concentrations of each.

The assignments of the free analytes are shown in Table 5.2. while Tables 5.3. and 5.4. show the change in chemical shift (Δδ) observed on complexation with each CD and the separation between signals arising from the individual enantiomers ($|\delta_R-\delta_S|$).

Table 5.2. ^{1}H NMR assignments of phenethylamines. For key to **1-9** see Fig. 5.2.

	H2'	H3'	H4'	H5'	H6'	H1	H2	R_2	2-CH_3
1	7.355					5.060	3.476	CH_3 2.699	1.062
2	6.862	7.245	-	7.245	6.862	4.904	3.230, 3.207	CH_3 2.693	-
3	6.871	7.217	-	7.217	6.862	4.951	3.416	CH_3 2.676	1.078
4	6.861	-	6.826	7.271	6.918	4.885	3.277, 3.085	-	-
5	6.847	-	6.812	7.254	6.901	4.906	3.249, 3.197	CH_2 3.071 CH_3 1.213	-
6	6.422	-	6.317	-	6.422	4.824	3.204, 3.133	CH 3.407 CH_3 1.258 CH_3 1.251	-
7	6.876	-	-	6.868	6.789	4.798	3.206, 3.094	-	-
8	6.865	-	-	6.878	6.789	4.811	3.196, 3.145	CH 3.407 CH_3 1.260 CH_3 1.244	-
9	7.275	CH_2 4.593	-	6.882	7.205	4.841	3.207, 3.128	Bu' 1.310	-

As representative spectra, Figure 5.3. shows the NMR spectra of uncomplexed etilefrine and oxedrine with their corresponding signals in the presence of β-CD and Ac-β-CD. In the case of etilefrine (**5**) the two aromatic doublets (H4' and H6') were distinguished with the aid of a 2D proton detected ^{1}H-^{13}C chemical shift correlation spectrum and substituent increment tables.

Figure 5.3. (a) The NMR signals for etilefrine HCl. Aromatic protons, H1 and CH_3 (R_2) in (a) no CD, (b) with β-CD and (c) with Ac-β-CD. EtAc is ethyl acetate impurity.

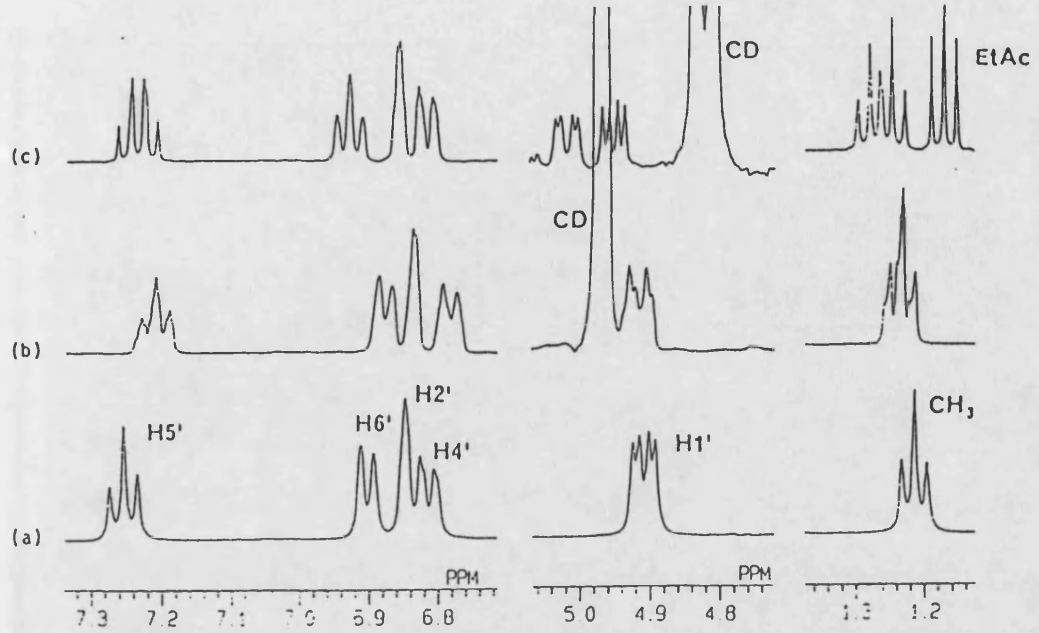


Figure 5.3b. The NMR signals for oxedrine HCl. Aromatic protons, H1, H2 and CH_3 (R_2) in (a) the absence of CD, (b) the presence of β-CD and (c) the presence of Ac-β-CD.

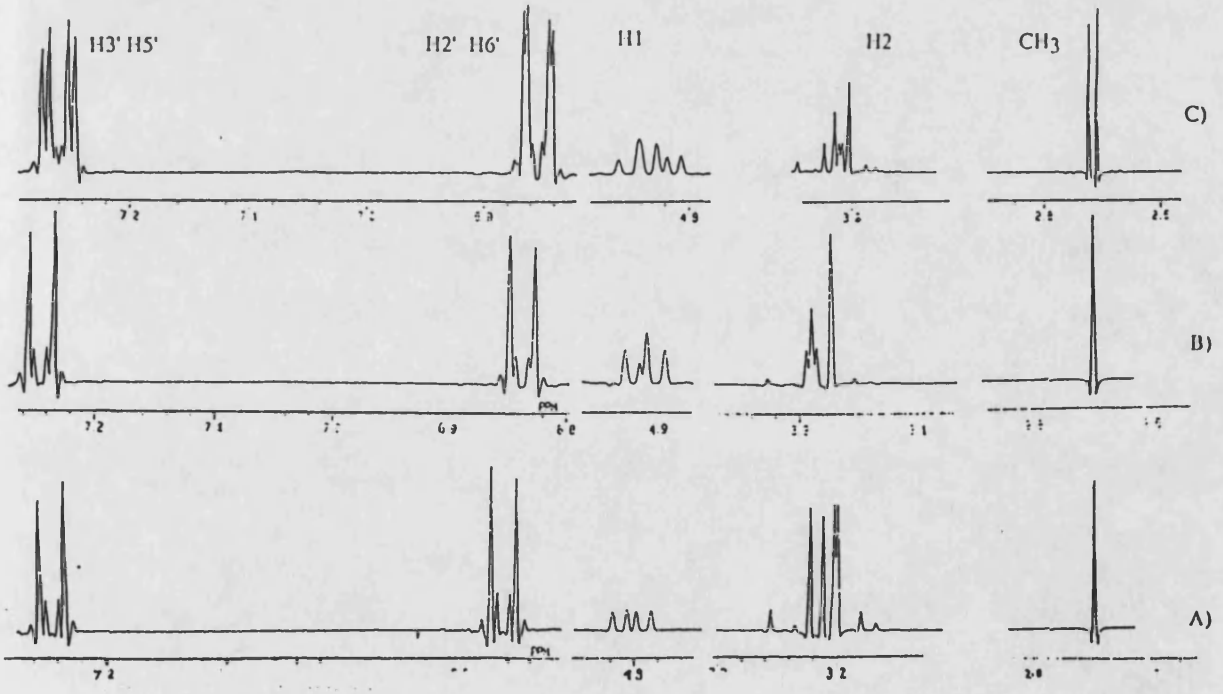


Table 5.3. Chemical shift changes (Δδ) in ^{1}H NMR spectra of various phenethylamines induced by β -cyclodextrin. The average change in chemical shift for both enantiomers is given with $|\delta_R - \delta_s|$ in parentheses. A dash in parentheses indicates no signal splitting and an asterisk indicates that full analysis was not possible.

	H2'	H3'	H4'	H5'	H6'	H1	H2	R_2	2-CH_3
1	-0.002 (*)					0.072 (-)	-0.096 (0.018)	CH_3 0.046 (-)	-0.027 (-)
2	0.000 (-)	-0.024 (-)	-	-0.024 (-)	0.000(-)	0.008 (-)	0.037 (*)	CH_3 0.010 (-)	-
3	-0.010 (0.005)	-0.058 (0.005)	-	-0.058 (0.005)	-0.010 (0.005)	0.086 (-)	-0.100 (0.017)	CH_3 0.052 (0.002)	-0.015 (0.003)
4	-0.013 (-)	-	-0.027 (-)	-0.040 (0.003)	0.023 (-)	0.000 (-)	-0.024 (0.013) -0.044 (0.009)	-	-
5	-0.015 (0.005)	-	-0.032 (*)	-0.053 (0.005)	-0.027 (-)	0.005 (-)	*	CH_2 * CH_3 0.016 (0.010)	-
6	-0.051 (0.006)	-	-0.045 (0.005)	-	-0.051 (0.006)	-0.003 (-)	-0.071 (-) 0.068 (-)	CH 0.014 (-) CH_3 0.021 (-) CH_3 0.023 (0.008)	-
7	-0.018 (-)	-	-	-0.041 (*)	-0.012 (-)	-0.002 (-)	-0.034 (-) -0.041 (-)	-	-
8	-0.012 (-)	-	-	-0.028 (-)	0.019 (-)	0.007 (-)	-0.051 (-) 0.024 (-)	CH 0.007 (-) CH_3 0.012 (-) CH_3 0.011 (-)	-
9	0.000 (-)	CH_2 0.000 (-)	-	-0.007 (-)	0.001 (-)	0.008 (-)	-0.033 (-) -0.005 (-)	Bu' 0.006 (-)	-

Table 5.4. Chemical shift changes (Δδ) in ^{1}H NMR spectra of various phenethylamines induced by *heptakis*(2,3-di-*O*-acetyl)β -cyclodextrin. $|\delta_R - \delta_s|$ shows the average change in chemical shift for eachenantiomer given in parentheses. A dash in parentheses indicates no signal splitting and an asterisk indicates that full analysis was not possible.

	H2'	H3'	H4'	H5'	H6'	H1	H2	R_2	2-CH_3
1	0.026 (*)					0.063 (0.040)	0.050 (0.036)	CH_3 0.041 (0.026)	0.016 (-)
2	0.015 (0.006)	-0.007 (0.003)	-	-0.007 (0.003)	0.015 (0.006)	0.030 (0.018)	0.001 (-) -0.003 (-)	CH_3 0.022 (0.007)	-
3	0.039 (0.021)	-0.010 (0.018)	-	-0.010 (0.018)	0.039 (0.021)	0.062 (0.046)	-0.042 (0.037)	CH_3 -0.043 (0.031)	0.019 (-)
4	0.009 (-)	-	-0.001 (0.006)	-0.004 (-)	0.023 (0.016)	0.038 (0.030)	0.043 (0.013) -0.008 (0.015)	-	-
5	0.011 (0.006)		0.006 (0.006)	-0.019 (0.016)	0.029 (0.018)	0.081 (0.067)	*	CH_2 * CH_3 0.034 (0.033)	-
6	-0.005 (-)	-	-0.011 (0.008)	-	-0.005 (-)	0.025 (*)	0.010 (0.004) 0.005 (0.032)	CH 0.039 (0.016) CH_3 0.029 (-) CH_3 0.034 (0.005)	-
7	-0.005 (-)	-	-	-0.026 (-)	-0.004 (-)	0.022 (*)	0.033 (-) -0.018 (-)	-	-
8	0.002 (-)	-	-	-0.012 (0.003)	0.006 (0.005)	*	0.003 (-) 0.019 (-)	CH 0.030 (0.016) CH_3 0.025 (0.008) CH_3 0.025 (0.008)	-
9	0.011 (-)	CH_2 0.004 (-)	-	0.002 (-)	0.010 (-)	0.014 (*)	0.032 (*)	Bu' 0.036 (0.015)	-

All the compounds show evidence of inclusion by β-CD with substantial changes in chemical shift (except salbutamol) caused by the presence of the oligosaccharide. In those compounds lacking a methyl group at C2, H1 showed very small changes in chemical shift, whereas in ephedrine and oxilofrine with the additional side-chain methyl group, H1 had a large value though not as large as H2. A contrasting pattern of values was observed on the addition of Ac-β-CD. In this case, the largest values of $\Delta\delta$ were observed for H1, although it could not be measured in all cases because the shifted signal was obscured by CD peaks. The other signals were shifted to a lesser extent than when β-CD was added and the direction of the shift differed in some cases. The aromatic signals showed a different pattern of shifts in most compounds e.g. in oxedrine and oxilofrine, with a *para*-hydroxy group, the *ortho*-protons had a smaller shift with β-CD but a larger one with the acetylated CD.

Chiral discrimination was much higher with Ac-β-CD. All compounds, except noradrenaline, had at least one signal that was split when this CD was added. The H1 signal had the highest $|\delta_R-\delta_S|$ values in the molecule in the instances where it was not obscured by CD signals and could be measured.

5.2.2. Δδ of β and Ac-β-CD Protons on Complexation with Phenethylamines.

Tables 5.5. and 5.6. show the chemical shifts experienced by the CD protons upon complexation with the various analytes. The H3" and H5" protons had the largest shifts (upfield) in both CDs, with the H3" and H5" shifts of β-CD being much larger than those of Ac-β-CD. The analyte aromatic rings induced a shielding effect due to their proximity to these inner CD protons thus indicating that complexation had occurred. H6" shifts of both CDs were also of considerable size suggesting the narrower (primary) end of each CD torus may have been involved in the complexation process.

Table 5.5. Δδ of β-CD protons upon complexation with phenethylamines. For key **1-9** see Fig. 5.2.

	H1"	H2"	H3"	H4"	H5"	H6"
1	-0.024	-0.007	-0.091	-0.021	-0.154	-0.058
2	-0.015	-0.003	-0.076	-0.010	-0.101	-0.038
3	-0.013	0.001	-0.063	-0.006	-0.108	-0.029
4	-0.011	-0.003	-0.044	-0.004	-0.055	-0.040
5	-0.027	-0.011	-0.064	-0.016	-0.081	-0.059
6	-0.019	-0.001	-0.068	0.010	-0.078	-0.054
7	-0.012	-0.005	-0.044	-0.006	-0.043	-0.037
8	-0.013	0.000	-0.054	-0.003	-0.076	-0.045
9	-0.006	-0.002	-0.032	-0.005	-0.019	-0.032

Table 5.6. Δδ of Ac-δ-CD protons upon complexation with phenethylamines. For key **1-9** see Fig. 5.2.

	2,3-di-O-acetyl	H1"	H2"	H3"	H4"	H5"	H6"
1	-0.007, -0.003	-0.012	-0.005	-0.016	-0.008	-0.016	-0.013
2	-0.013, -0.005	-0.014	-0.006	-0.027	-0.013	-0.027	-0.023
3	-0.002, 0.001	-0.006	0.001	-0.009	0.011	-0.006	0.010
4	-0.002, -0.001	-0.007	-0.003	-0.004	-0.002	-0.018	-0.011
5	-0.018, -0.010	-0.027	-0.015	-0.021	-0.012	-0.090	-0.041
6	-0.011, 0.000	-0.021	-0.009	-0.006	0.000	-0.059	-0.018
7	-0.017, -0.015	-0.022	-0.018	-0.016	-0.008	-0.032	-0.036
8	-0.013, -0.004	-0.016	-0.006	-0.010	-0.005	-0.041	-0.026
9	-0.010, -0.003	-0.004	-0.001	-0.004	0.002	-0.005	-0.006

5.2.3. Analyte Complexation with α and γ-CD.

Although neither of these CDs had provided any enantio-resolution for the phenethylamines when employed in the HPLC experiments, their interaction with several of these analytes was examined. The NMR conditions chosen were those which had been used for the β-CD and Ac-β-CD work (section 5.2.1.).

None of the analytes tested with γ-CD showed any significant changes in chemical shift. The large size of the γ-CD cavity would mean that compounds with only one planar aromatic ring would be unable to obtain a 'tight-fit' inside the γ-CD cavity. Their motion would be relatively unrestricted within the CD torus and they would be unlikely to favour this environment over that of the bulk solvent with the result that interaction with the CD would be weak. It was therefore unsurprising that no change in chemical shift of either the analyte protons or the inner CD protons was observed. Similar results were seen in the presence of α-CD. In this case the rather small nature of the CD cavity (4.5 - 6.0 Å) may have proved unfavorable for inclusion complexation with the substituted phenyl rings of the compounds. However, other workers have noted shifts of the inner protons of α-CD upon interaction with e.g. benzoic acid (Bergeron *et al.* 1978) and *p*-iodoaniline (Wood *et al.* 1977). It may be, that the more extensive nature of the phenyl substituent in these compounds sterically hinders complexation.

5.2.4. Interpretation of Results.

The two CDs (β- and Ac-β-CD) had different overall effects on the NMR spectra of the phenethylamines as shown by chemical shift changes and signal splitting arising from diastereomeric complexation with the enantiomeric guest molecules. Both CDs demonstrated inclusion of phenethylamines but β-CD discriminated poorly between enantiomers and Ac-β-CD on the whole resolved the enantiomers extremely well. Neither α-CD nor γ-CD were found to induce significant chemical shifts, which could have been attributed to the dimensions of the cavities in relation to the overall size of the analytes.

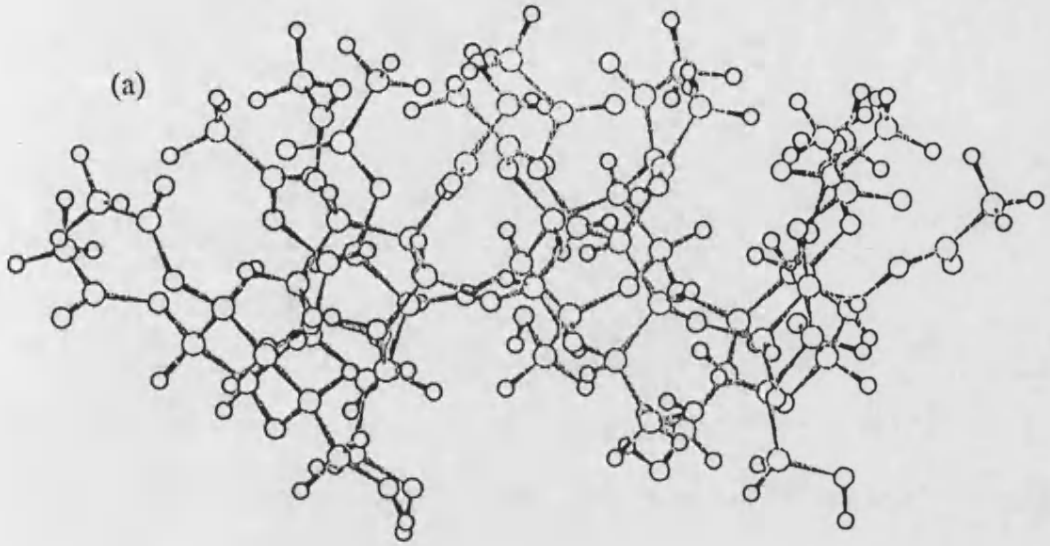
The larger chemical shifts of H3" and H5" in β-CD (compared to those in Ac-β-CD) indicate that this CD forms stronger inclusion complexes via a 'tighter fit', but not necessarily of a better enantio-discriminating nature, as seen from the lack of signal splitting. Relative to H5", the H6" signal shifts were higher with Ac-β-CD than β-

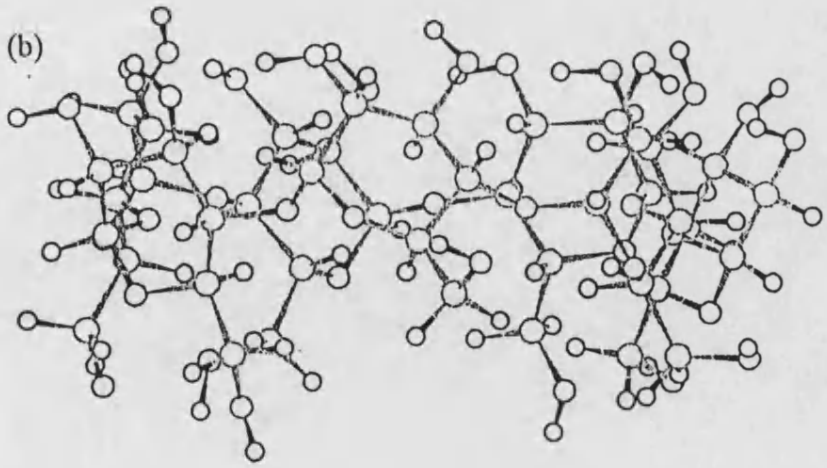
CD. Conformational changes of Ac-β-CD ascertained with molecular modelling, show that the secondary CD rim increased in size relative to the parent CD and that the primary opening became smaller (Fig. 5.4.). This was probably due to a total removal of the CD intramolecular hydrogen bonding between the OH (2) and OH (3) groups, which normally helps to stabilise the torous (Onda *et al.* 1988) and steric interaction between the acetyl groups. Such conformational flexibility was generally thought to occur only with *per*-derivatised CDs (Harata *et al.* 1984 and Ventura *et al.* 1994). Other workers have stated that a significant shift of H6" protons (along with H5") indicates a deeper analyte penetration of the CD cavity (Rekharsky *et al.* 1994), which would explain the larger H6" shifts with Ac-β-CD found in this work. The smaller Ac-β-CD H3" shifts arise from a widening of the secondary opening that reduces the analyte interaction at that point.

Changes in analyte chemical shift provide evidence of inclusion by both oligosaccharides, although the Δδ values for salbutamol are small or insignificant on complexation with Ac-β-CD. The presence of β-CD tended to cause larger shifts in the aromatic region compared to Ac-β-CD and produced little or no splitting of the signals. On the other hand, Ac-β-CD split aromatic signals in several compounds, most notably in oxilofrine, norfenefrine and etilefrine.

Mainly upfield shifts (negative Δδ values) were observed in the aromatic region of the phenethylamine spectra on complexation with either CD and are consistent with inclusion of this part of the molecule in the hydrophobic cavity of the macromolecule. Downfield shifts in the H6' signal of some *N*-(2',4'-dinitrophenyl)amino acids, together with circular dichroism data, have been interpreted as arising from inclusion complexes where the guest molecule is tilted in the cyclodextrin cavity (Li and Purdy 1992) and are consistent with theoretical calculations on the alignment of guest and host dipole moments (Kitigawa *et al.* 1990). Notable downfield shifts occurred for individual aromatic resonances of some phenethylamines and may be indicative of such tilting, for example, the H6' resonance is shifted downfield on complexation with β-CD in norfenefrine while other signals move upfield. Whilst the overall

Fig. 5.4. Molecular models of (a) Ac-β-CD and (b) β-CD. Calculated using the Discovery package (see section 2.5.).



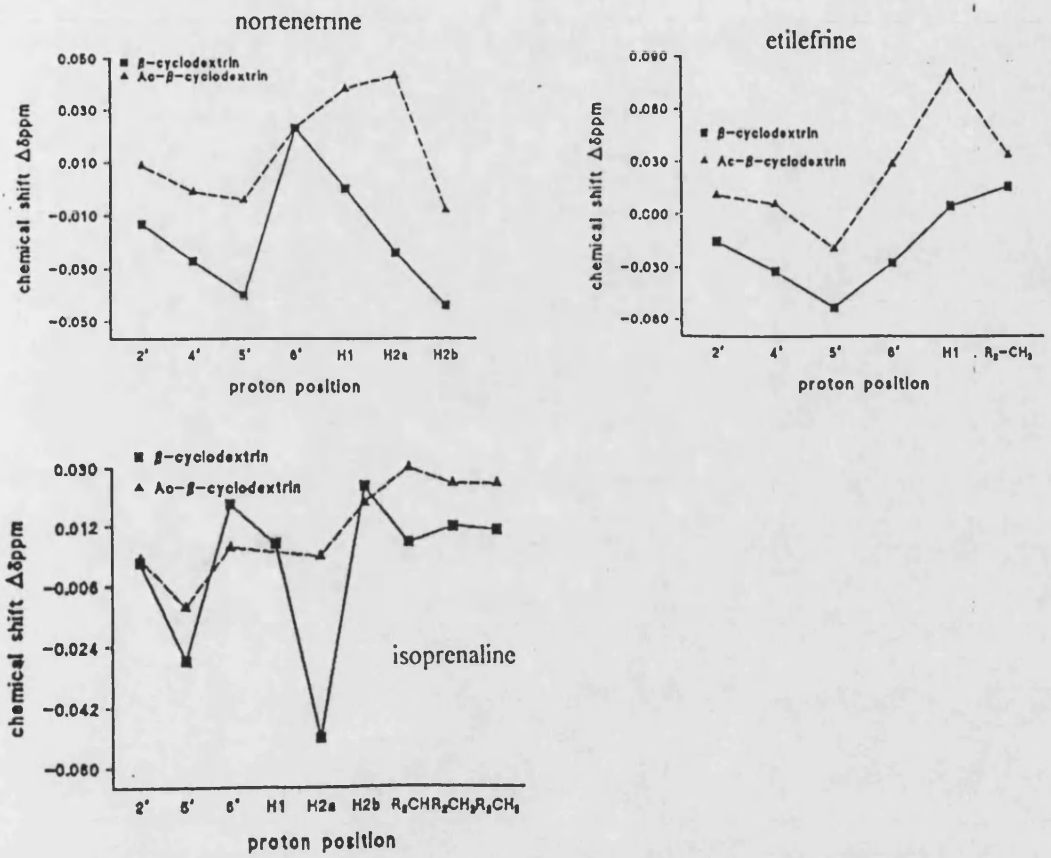


pattern of aromatic chemical shifts is similar with both CDs, in the presence of Ac-β-CD, the H6' signal for norfenefrine, etilefrine and isoprenaline shifted downfield to a greater extent than the other aromatic signals (see Fig. 5.5.).

Side-chain interactions with the CD, presumably at its surface, are apparently also important for complexation, although dependant on derivatisation, and the alkyl values are larger than for the aromatic signals. The signals of H2 and R_2 are significantly shifted in the presence of either CD, and often have large values of $|\delta_R - \delta_s|$. However, the H1 resonance is significantly shifted only by Ac-β-CD for compounds without a methyl group at C2. Ac-β-CD gives a large splitting of the H1 resonance in those compounds where the signal is not obscured but in the presence of β-CD no doubling is observed. These results could be explained by different hydrogen bonding interactions between each CD and the guest side-chain. Both hydroxyl and acetyl groups on the CD rim could act as hydrogen bond acceptors for the amine NH thus causing chemical shift changes in the guest (although of variable direction) with both CDs. The downfield shifts of the H1 resonance noted with the derivatised CD may be due to hydrogen bonding between the guest hydroxyl group at C1 and the carbonyl oxygen of the macrocyclic acetyl groups. Presumably the structure of the inclusion complex with β-CD does not have the correct geometry for a similar interaction with the secondary hydroxyl groups of this CD.

In the presence of β-CD, ephedrine and oxilofrine with the 2-Me group also show a large shift of the H1 resonance which could be explained by the side-chain methyl group forcing a conformational rearrangement such that a hydrogen bond can be accommodated. Differences in hydrogen bonding patterns could also explain why Ac-β-CD is better than the parent CD at discriminating between phenethylamine enantiomers, as shown by the generally larger values of $|\delta_R - \delta_S|$ obtained with the former oligosaccharide.

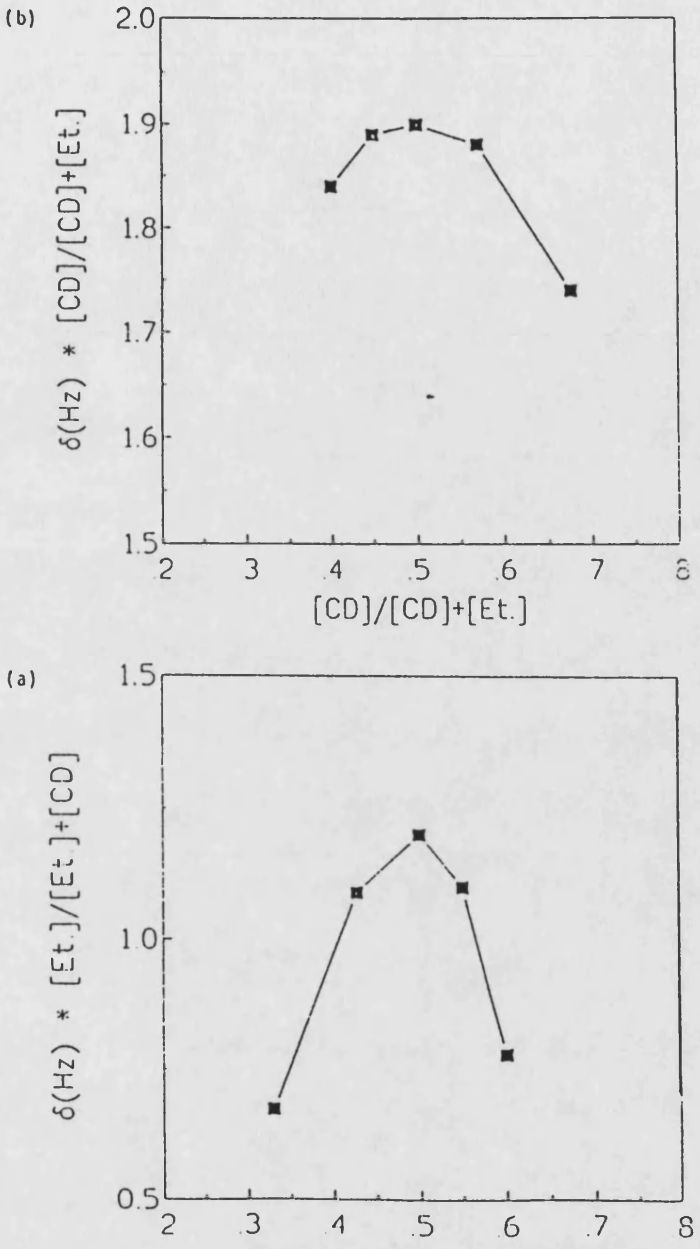
Fig. 5.5. Effect of β-CD and Ac-β-CD on Δδ of norfenefrine, etilefrine and isoprenaline. See text for explanation.



5.2.4. Calculation of Stoichiometry and Association Constant, (K_f).

A Job (continuous variation) plot (see section 5.1.5. for a description of the method and section 2.4.3. for details of the experimental procedure) was used to determine the stoichiometry of each CD complex in the case of etilefrine HCl (Fig. 5.6.). Selected data for guest or host are shown but other signals reflected the same pattern with a peak at 0.5 showing that a 1:1 complex was formed with both CDs. It is assumed that the other compounds in the series also formed complexes with this stoichiometry due to their lack of a second aromatic ring in the molecules.

Fig. 5.6. Representative Job plots. Complexation between etilefrine HCl and (a) β-CD (H1" resonance) and (b) Ac-β-CD (H3" resonance).



Four solutions in 0.1M deuterated phosphate buffer, pH 4.5 with molar ratios (guest:host) of 1:2.33, 1:3.0, 1:4.0 and 1:5.7 were prepared to determine the K_f between etilefrine HCl and β-CD and Ac-β-CD. The graphical Foster-Fyfe method was used to determine K_f (see section 5.1.3. for details). However, plots of $1/\Delta\delta_{obs}$ *vs* 1/[CD] were not linear using CH_3 or α-H on the guest or H1" on the host. It was not possible therefore to evaluate K_f using NMR in this instance.

5.3. NMR Investigation of Propranolol Analogues.

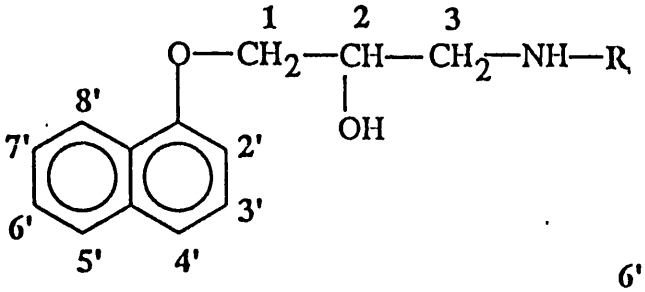
HPLC (section 3.2.) and FSCE (section 4.3.) studies had proven that β-CD, HE-β-CD, Me-β-CD, Ac-β-CD and peracetyl-β-CD were able to resolve the enantiomers of various propranolol analogues, with FSCE providing the greatest enantio-separation values. Consequently these CDs were used for the NMR investigations to understand more fully those 'host-guest' interactions involved. As stated previously (section 5.2.), it was not possible to examine the peracetyl-β-CD:analyte complexes due to the high level of deuterated organic solvent required for this CD's dissolution, which in turn prevented the observation of any significant δ values. However those FSCE buffer conditions which gave rise to the highest observed chiral separations were replicated in the NMR experiments using the appropriate deuterated solvents (D_2O and CD_3OD) with a host:guest molar ratio of approximately 1:1.

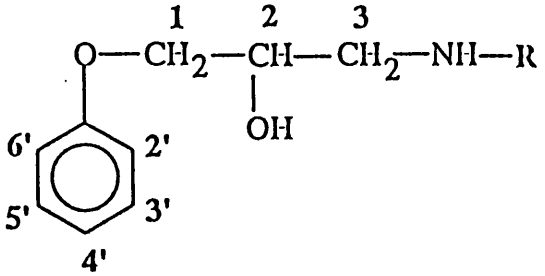
5.3.1. Analyte Complexation with β-CD.

Greatbanks and Pickford 1987 examined propranolol's complexation with β-CD and found that mostly downfield shifts occurred for the proton signals of guest molecule protons. The result for propranolol obtained in the present work (compound **1**; Table 5.7., see p.158) is in agreement, with H2', H3' and H8' having the largest downfield shifts of the aromatic protons while the side chain protons showed generally lower shifts (assignments were readily completed upon inspection and via the aforementioned reference). Fig. 5.7. (see p.157) shows the labelling scheme used for compounds **1-6**. NOE measurements suggested that the guest adopted a tilted conformation within the β-CD cavity, with irradiation at the β-CD H5" giving a positive NOE for the guest H5' and H8' signals whilst irradiation of the β-CD H3" gave an enhancement at only H8' of the guest. Further, Greatbanks and Pickford saturated the isopropyl site and found no intensification for any of the CD signals suggesting that it does not occupy the CD torous. However, in the work described

here, the isopropyl group displayed a strong shift upon complexation with the CD, indicating some interaction with the oligosaccharide.

Fig. 5.7. NMR labelling scheme for compounds 1-6.





R= *i*-propyl (compound **1**)
R = *t*-butyl (compound **2**)
R = ethyl (compound **3**)
R = CONH-ethyl (compound **6**)

R = *i*-propyl (compound **4**)
R = *t*-butyl (compound **5**)

With all the guest R groups demonstrating significant downfield chemical shifts, where they could be readily measured, they apparently underwent some conformational change upon interaction of their aromatic moiety with β-CD.

Signal splitting for compounds **1-3** i.e. those analytes which possessed a naphthyl ring, occurred at the methylene group in position 3 (in compound **6** the signal was obscured) and also in position R for compounds **3** and **6**. Such signal splitting is indicative of differential interaction between the guest enantiomers with the host macrocycle. Compounds **4** and **5**, which possessed only a single aromatic ring, displayed a different pattern of aromatic shifts to the other analytes. These shifts indicated inclusion into the CD cavity but were generally smaller than the $\Delta\delta$ values observed for those analogues containing a naphthyl ring.

Observation of the β-CD protons (Table 5.8., see p.158), assigned with the aid of literature values e.g. Casy and Mercer 1988), showed that all the analytes caused a significant upfield shift of the H3" signal. H5" shifts were weaker, especially with

compounds **1, 2, 4** and **5**, suggesting less shielding of this proton, perhaps from a more shallow insertion into the CD cavity. H6", positioned at the primary CD end, generally had a much smaller Δδ than H5". The lower CD Δδ seen for compound **6** indicated a weaker complexing interaction.

Table 5.7. Δδ of compounds 1-6 upon the addition of β-CD. NMR conditions: 30% CD_3OD - phosphate buffer, 0.05 M, pH 3.0.

	2'	3'	4',6'	7'	5'	8'	1	2	3	R	R
1	0.095	0.098	0.059	0.059	0.042	0.088	0.080	*	0.047 (0.004)	CH *	$(CH_3)_2$ 0.065
2	0.092	0.096	0.061	0.061	0.040	0.087	0.083	*	0.051 (0.008)	$(CH_3)_3$ 0.065	-
3	0.098	0.102	0.055	0.055	0.031	0.089	0.066	*	0.072 (0.005)	CH_2 0.071 (0.005)	CH_3 0.069
4	0.060	0.061	0.060	-	0.061	-	0.066	0.096	0.056	CH 0.060	$(CH_3)_2$ 0.071
5	0.054	0.060	0.054	-	0.060	-	0.072	0.112	0.030	$(CH_3)_3$ 0.068	-
6	0.093	0.089	0.034	0.034	0.017	0.072	*	*	*	CH_2 0.069	CH_3 0.066 (0.002)

* signal obscured.

Table 5.8. Δδ of β-CD protons upon complexation with compounds (1-6). NMR conditions as stated in Table 5.7.

Analyte	1"	2"	3"	4"	5"	6"
1	-0.011	-0.006	-0.009	-0.002	-0.002	-0.001
2	0.001	-0.013	-0.021	0.005	0.004	0.000
3	-0.007	-0.013	0.001	-0.007	-0.007	0.001
4	-0.012	-0.007	-0.010	-0.002	-0.002	-0.002
5	-0.043	-0.032	-0.033	-0.025	-0.024	-0.006
6	-0.017	-0.014	-0.012	-0.008	-0.008	-0.004

5.3.2. Analyte complexation with Ac-β-CD.

The analyte chemical shift values induced by Ac-β-CD (Table 5.9., see p.158) were both up and downfield for the aromatic protons of compounds **1-3** and **6** (naphthyl R' group), whilst those of compounds **4** and **5** (phenyl R' group) moved only downfield, suggesting a difference in their inclusion position. Virtually all the alkyl proton resonances for compounds **1-6** moved to lower-field with position 2 having the

Table 5.9.Δδ of compounds 1-6 upon the addition of Ac-β-CD.

NMR conditions as stated in Table 5.7.

Analyte	2'	3'	4',6'	7'	5'	8'	1	2	3	R	R
1	0.005	0.001	-0.001	-0.001	0.002	0.002	0.004	0.020	*	CH 0.009	$(CH_3)_2$ 0.012
2	-0.004	-0.002	-0.001	-0.001	-0.005	-0.005	-0.009	0.017	0.010	$(CH_3)_3$ 0.013	-
3	0.002	0.001	-0.014	-0.014	-0.005	-0.003	0.001	0.007	0.003	CH_2 0.008	CH_3 0.009
4	0.004	0.002	0.004	-	0.002	-	0.008	0.005	0.019	CH 0.006	$(CH_3)_2$ 0.010
5	0.004	0.002	0.004	-	0.002	-	0.009	0.006	0.025	$(CH_3)_2$ 0.013	-
6	-0.017	-0.016	-0.020	-0.020	-0.015	-0.024	*	*	-0.009	CH_2 0.008	CH_3 0.008

* signal obscured.

Table 5.10. Δδ of Ac-β-CD protons upon complexation with compounds (1-6).

NMR conditions as stated in Table 5.7.

Analyte	1"	2"	3"	4"	5"	6"	Ac	Ac
1	-0.021	-0.020	-0.020	-0.013	-0.013	-0.015	-0.012	-0.015
2	-0.023	-0.020	-0.021	-0.014	-0.012	-0.015	-0.012	-0.015
3	0.011	0.004	0.001	0.003	0.004	0.005	-0.010	-0.013
4	-0.032	-0.023	-0.024	-0.014	-0.013	-0.017	-0.008	-0.008
5	-0.028	-0.024	-0.028	-0.015	-0.014	-0.022	-0.008	-0.008
6	-0.026	-0.027	-0.031	-0.012	-0.012	-0.024	-0.006	-0.008

highest value for compounds **1-3** and position 3 the largest chemical shift for compounds **4** and **5**. No signal splitting was observed for any of the compounds.

The Ac-β-CD protons were assigned by inspection, with the aid of a 2D ^1H-^1H COSY and by comparison with β-CD. On complexation with compounds **1-6** (Table 5.10.) H5" and H6" had weaker shifts than H3" in the presence of compounds **1-3** and **6.** H1", H2" and H4", located on the 'outside' of the CD torous displayed stronger upfield shifts than the 2, 3-acetyl groups on the secondary rim.

5.3.3. Analyte complexation with HE-β-CD.

The aromatic protons of compounds **1-5** showed strong upfield shifts indicative of shielding when complexed with HE-β-CD although the H2', H3' and H8' aromatic protons of compounds **1-3** had considerably smaller shifts than the others (Table 5.11., see p.162). The aromatic protons of compounds **4** and **5** showed very similar shifts with little variation between the individual values. The methylene group in position 3 had the largest upfield shift of the alkyl protons for compounds **1-3** and the chiral proton (position 2) had the smallest shifts in all cases (obscured in compound **6**). Signal splitting was apparent in several instances, most notably of R in **3** and **6** (Fig. 5.8., see p.161) and of the methylene groups in positions 1 and 3 for compounds **2, 3** and **5**.

Interpretation of the HE-β-CD signals was based on observation and on the assignment of *heptakis*(2, 6-di-*O*-ethyl)β-CD by Hirayama *et al.* 1993 and of other alkylated β-CDs by Meier-Augenstein *et al.* 1992. All the HE-β-CD protons were shifted upfield (Table 5.12., see p.162) in the presence of compounds **1-6**, with the H3" and H5" protons showing the largest shifts, indicative of analyte inclusion. Unfortunately the latter two signals overlapped preventing individual assignment. Compounds **4, 5** and **6** induced

lower CD shifts than compounds **1-3**. The hydroxyethyl signals displayed stronger shifts than H6", suggesting preferential analyte interaction at the wider secondary rim of the CD torous. The hydroxyethyl group signals were broad because of the non-specific substitution pattern (MS = 0.6). An average value of their changing chemical shift is given in Table 5.12.

Fig. 5.8. Signal splitting of compounds 3 and 6 in presence of HE-β-CD. (a) **6** plus HE-β-CD, (b) **6** uncomplexed, (c) **3** plus HE-β-CD and (d) **3** uncomplexed. NMR conditions: 30% CD_3OD - phosphate buffer, 0.05 M, pH 3.0.

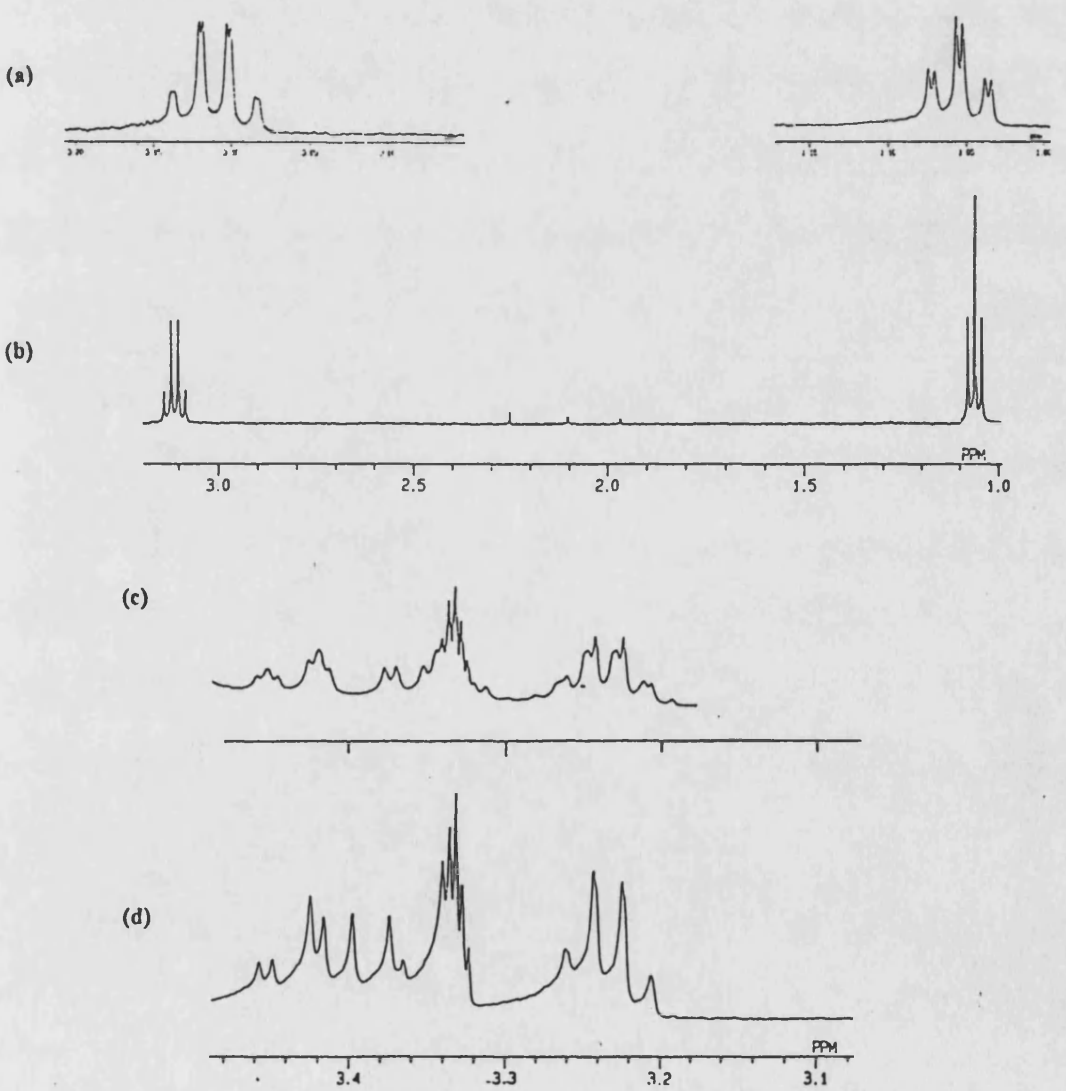


Table 5.11. Δδ of compounds 1-6 upon the addition of HE-β-CD.

NMR conditions: 30% CD_3OD - phosphate buffer, 0.05 M, pH 3.0.

Analyte	2'	3'	4',6'	7'	5'	8'	1	2	3	R	R
1	-0.032	-0.041	-0.105	-0.105	-0.151	-0.050	-0.062	-0.031	-0.143	CH -0.075	$(CH_3)_2$ -0.097
2	-0.049	-0.065	-0.118	-0.118	-0.161	-0.059	-0.082	-0.032	-0.133 (0.010)	$(CH_3)_3$ -0.104	-
3	-0.029	-0.047	-0.107	-0.107	-0.149	-0.040	-0.056	-0.021	-0.098 (0.005)	CH_2 -0.086 (0.005)	CH_3 -0.095
4	-0.108	-0.110	-0.108	-	-0.110	-	-0.104	-0.057	-0.090	CH *	$(CH_3)_2$ -0.085
5	-0.093	-0.099	-0.093	-	-0.099	-	-0.083 (0.004)	-0.050	-0.092	$(CH_3)_3$ -0.074	-
6	0.029	0.033	-0.028	-0.028	-0.069	0.009	*	*	*	CH_2 0.002 (0.002)	CH_3 -0.006 (0.005)

* signal obscured.

Table 5.12. Δδ of HE-β-CD protons upon complexation with compounds (1-6).

NMR conditions: 30% CD_3OD - phosphate buffer, 0.05 M, pH 3.0.

Analyte	1"	2"	3"	4"	5"	6"	OH-CH_2-CH_2-
1	-0.067	-0.071	-0.066	-0.055	-0.045	-0.005	-0.097
2	*	*	*	-0.050	-0.037	-0.009	-0.104
3	-0.090	-0.091	-0.088	-0.064	-0.042	-0.064	-0.104
4	-0.077	-0.079	-0.074	-0.055	-0.055	-0.055	-0.093
5	-0.090	-0.091	-0.088	-0.064	-0.042	-0.064	-0.089
6	-0.075	-0.082	-0.072	-0.043	-0.035	-0.053	-0.093

* Signal obscured.

5.3.4. Analyte Complexation with Me-β-CD.

All changes in chemical shifts were upfield (shielded) for compounds **1-6** when Me-β-CD was added (Table 5.13.), with H2', H3' and H8' of compounds **1-3** and **6** showing noticably smaller shifts than the other aromatics. There was little variation in the aromatic shifts of compounds **4** and **5**. The alkyl signal at position 2 (chiral proton) was more strongly shielded in compounds **4** and **5**, which occurred with all the CDs (where measurable) except Ac-β-CD. All the R groups were strongly shielded but no splitting was observed.

Table 5.14., see p.164, shows the Me-β-CD proton shifts induced by compounds **1-6**, all of which were upfield except for H1", which showed clear downfield movement. H3" and H5" overlapped but still possessed the largest shifts from analyte inclusion. The high degree of substitution (DS = 1.8) allowed a relatively clear assignment of the Me-β-CD signals from inspection and via other references (Yamamoto *et al.* 1987 and Inoue *et al.* 1986).

Table 5.13. Δδ of compounds 1-6 upon the addition of Me-β-CD.

Analyte	2'	3'	4',6'	7'	5'	8'	1	2	3	R_1	R_1
1	-0.067	-0.090	-0.127	-0.127	-0.157	-0.085	*	-0.078	*	CH *	$(CH_3)_2$ -0.123
2	-0.075	*	-0.163	-0.163	-0.190	-0.083	*	-0.076	*	$(CH_3)_3$ -0.127	-
3	-0.073	-0.064	-0.141	-0.141	-0.184	-0.086	*	-0.086	*	CH_2 -0.120	CH_3 -0.124
4	-0.142	-0.141	-0.142	-	-0.141	-	*	-0.105	*	CH *	$(CH_3)_2$ -0.108
5	-0.129	-0.140	-0.129	-	-0.140	-	*	-0.112	*	$(CH_3)_3$ -0.110	-
6	-0.091	-0.096	-0.166	-0.166	-0.124	-0.112	*	*	*	CH_2 -0.115	CH_3 -0.129

* signal obscured.

With compounds 4 and 5 the Me-β-CD H3" was noticably more strongly shielded than H5". The H6" protons did not display significantly different chemical shifts to

those of the external protons. Interaction with the secondary rim seems then to be favoured with this CD derivative.

Table 5.14. Δδ of Me-β-CD protons upon complexation with propranolol analogues (1-6). NMR conditions: 30% CD_3OD - phosphate buffer, 50 mM, pH 3.0.

Analyte	1"	2"	3"	4"	5"	6"	Me-2	Me-3
1	0.144	0.142	0.140	0.162	0.164	0.146	-0.134	-0.126
2	-0.114	-0.113	-0.136	-0.131	-0.137	-0.113[a]	-0.133	-0.129
3	-0.113	-0.113	-0.130[b]	-0.138	-0.130[b]	-0.110	-0.136	-0.133
4	-0.116	-0.119	-0.133	-0.127[c]	-0.116	-0.118	-0.131	-0.127
5	-0.123	-0.123	-0.145	-0.138	-0.120	-0.120	-0.137	-0.126
6	-0.116	-0.122	-0.120	-0.129	-0.128	-0.113	-0.133	-0.128

[a] signal overlap with Me-2. [b] signal overlap between 3" and 5" protons. [c] signal overlap with Me-3.

5.3.5. Comparison of Results for Propranolol Analogues.

The direction of analyte chemical shifts i.e. whether up or downfield was dependant on the CD employed; β-CD caused deshielding of the aromatics for compounds **1-6**, Me-β-CD caused upfield shifts, HE-β-CD did likewise (except for compound **6**) and Ac-β-CD had a more varied effect. The overall magnitude of the aromatic and alkyl chemical shifts for compounds **1-6** was greatest with Me-β-CD and decreased in the order HE-β-CD, β-CD and Ac-β-CD, whilst this actual pattern of signal shifts was similar for compounds **1-3** and **6** but unlike that of compounds **4** and **5** (Fig. 5.9., see p.166). Analytes bearing a single aromatic ring (compounds **4** and **5**) are likely to induce smaller CD shifts than analytes with a naphthyl ring, perhaps due to the fact that the phenyl ring has a 'looser fit' within the β-CD type cavity (Technicol 1992).

The guest proton alkyl signals in positions 1, 2 and 3 were markedly shifted in every case and along with the large R chemical shifts, point to the influences of hydrogen bonding and a likely conformational rearrangement of the guests during the complexation mechanism. Inoue *et al.* 1987 and Uccello-Barretta 1993 have also

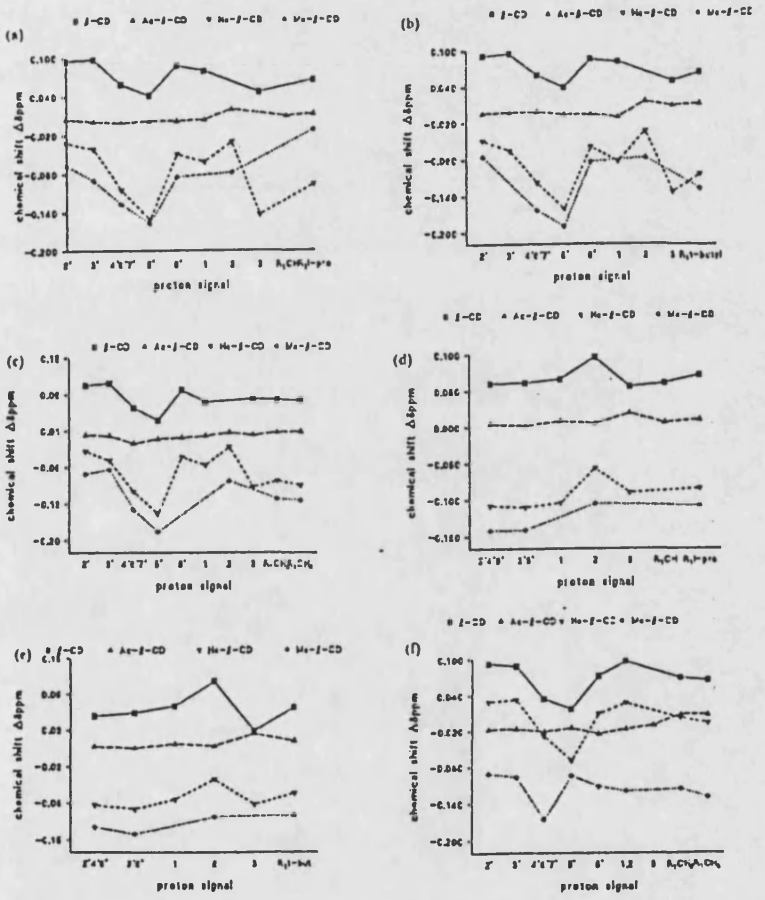
reported structural changes in the guest molecule upon inclusion with CDs. Analyte signal splitting (compounds **1, 2, 3** and **6**) was only seen with β-CD and HE-β-CD, mostly in the 3 and R positions. Interestingly, these were the only CDs used which can hydrogen-bond donate from their 2 and 3 positions. Clearly then, the alkyl chains and the terminal R groups are experiencing different chemical environments and must be important in the complexation process. This is in contrast to the result of Greatbanks and Pickford 1987, who found that the *i*-propyl group of propranolol (**1**) underwent a negligable chemical shift in the presence of β-CD. However their work was performed in pure D_2O and it is clear that solvent differences can have a profound effect upon the geometry and relative stabilities of various conformations (Sherrod 1992).

In all the complexes except those with β-CD, H6" experienced a significant change in chemical shift compared to H5". Widening of the secondary rim, as purported in section 5.2., would allow a deeper penetration of the guest mlecules and explain the relatively higher H6" signals seen with Ac-β-CD. Me-β-CD and HE-β-CD also displayed significant H6" shifts and therefore it appears that derivatisation has allowed a deeper analyte penetration. Yamashoji *et al.* 1992 attributed the relative H5"-H6" shift magnitude to analyte interaction (alanine β-naphthylamine) at the narrow rim of Ac-β-CD, where the naphthyl ring approached the H6" protons. However they failed to account for the sizable chemical shifts seen in their analyte's alkyl chain, now directed away from the CD, which appears inconsistent with their proposed mode of inclusion.

Chemical shift changes were largest with the derivatised groups of Me-β-CD and decreased successively for HE-β-CD and Ac-β-CD in the presence of compounds **1-6**. When hydrogen bonding occurs, those hydrogens most directly attached to the atoms involved are likely to experience the greatest change in electron distribution and hence the largest chemical shift. The methyl protons on Me-β-CD are two bonds removed from an oxygen atom, those of Ac-β-CD are four bonds removed and the protons of HE-β-CD are three bonds distant from a site of possible hydrogen

bonding, which may explain the relative Δδ's, assuming hydrogen bonding at these points.

Fig. 5.9. Δδ pattern of compounds 1-6 upon complexation with CDs. (a) compound **1**, (b) **2**, (c) **3**, (d) **4**, (e) **5** and (f) compound **6**.



It seems then that all the analytes approached each CD torus from the wider secondary rim and entered the CD cavity, whereupon their alkyl chains interacted with those groups on the secondary CD rim, presumably via hydrogen-bonding and steric forces. The three modified CDs displayed increased H6" chemical shifts, which was linked to a conformational change of the torus that allowed deeper penetration of the analytes into the cavity.

5.4. NMR Investigation of Mequitamium.

Prior to any complexation studies with the various CDs it was necessary to assign the NMR spectrum of mequitamium.

5.4.1. Determination of Mequitamium Structure.

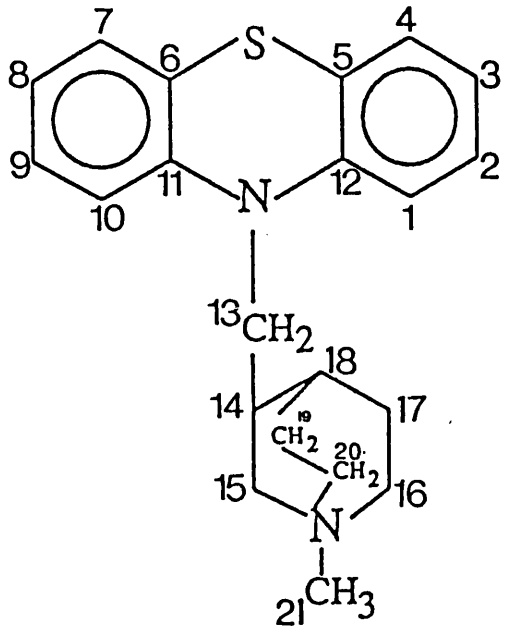
The structure of mequitamium was confirmed using the experimental techniques described in section 2.4.4. Table 5.15 gives the ^{1}H and ^{13}C-NMR signals assigned according to the numbering scheme of the compound as depicted in Fig. 5.10.

Table 5.15. ^{1}H and ^{13}C-NMR chemical shifts of Mequitamium.

Nuclei Position (see Fig. 5.6.)	$_{13}C$ Chemical Shift (ppm)	^{1}H Chemical Shift (ppm)
1/10[a]	116.46	7.24 (m)
2/9[a]	127.50	7.24 (m)
3/8[a]	123.04	7.01 (split t)
4/7[a]	127.76	7.24 (m)
5/6[a]	124.86	-
11/12[a]	144.93	-
13 [d]	59.56	13a 3.47 (m), 13b 3.12 (q)
14	31.15	2.59 (quintet)
15[c]	48.10	4.06 (dd), 4.12 (dd)
16/20[b]	55.77/55.61	3.35 (m)
17/19[a]	19.20	1.82 (m)
18	21.21	2.13 (bd)
19/17[a]	24.52	1.93 (m)
21	51.24	2.89 (s)

[a] Overlapping signal due to molecular symmetry.[b] Signals not assigned to respective nuclei. [c] Calculated according to equation 2.1. [d] Overlap with 16/20.

Fig. 5.10. NMR Labelling Scheme Adopted for Mequitamium.

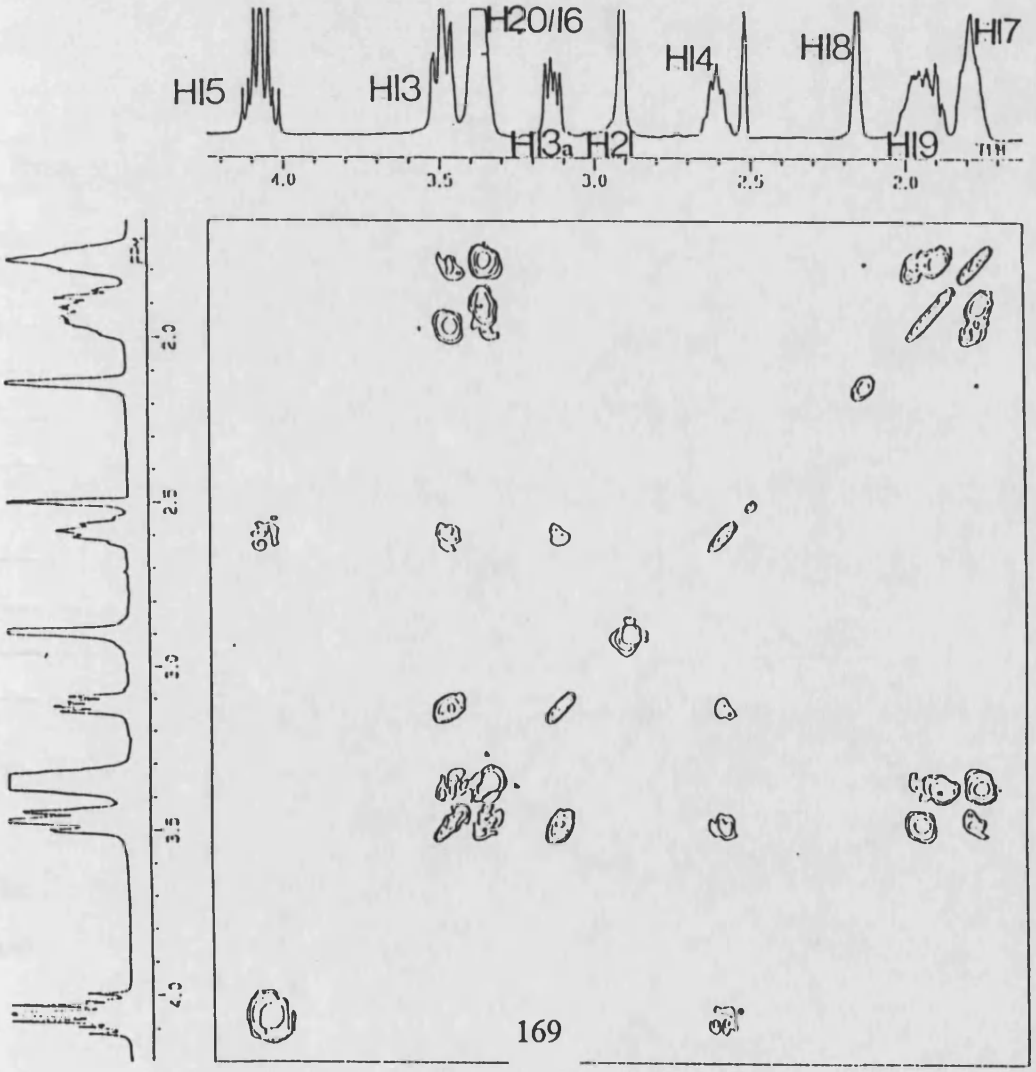


Initially, a broad-band decoupled ^{13}C spectrum and two DEPT experiments were performed to determine the bonding types of each carbon atom i.e. whether quaternary, CH, CH_2 or CH_3. A normal ^1H spectrum then enabled the direct identity of some signals by examination of the peak integral sizes and the expected splitting patterns (e.g. CH_3 - a sharp singlet with a peak integral proportional to that of three protons could only belong to the methyl group on carbon 21). A ^1H-^{13}C COSY (Correlation Spectroscopy) experiment and comparison with literature data on similar molecules allowed further signal assignments. ^1H splitting patterns are given in brackets in Table 5.15 e.g. (m) designates a multiplet, (t) is a triplet, (s) is a singlet etcetera.

Finally a ^1H-^1H COSY and data from 'Specinfo' (a computerised NMR database operated by Daresbury Laboratory) provided unequivocal proof for all the signals except as stated in Table 5.15. For example, the ^1H quintet signal seen at 2.6 ppm (Fig. 5.11.) was shown to belong to the proton on carbon number 14 (the chiral centre) and not carbon number 18 (the only other non-aromatic CH bonded car-

bon) by virtue of the cross peaks arising between itself and carbon number 15 (itself already distinguished from carbon 13 due its further downfield position caused by greater electron withdrawal of the attached quaternary nitrogen group). It would not be possible to observe such crosspeaks between carbons 15 and 18 due to the structure of the molecule. A portion of the mequitamium ^1H-^1H COSY is depicted in Fig. 5.11., showing how coupled resonances produce off diagonal cross peaks, the intensity of which relate to the distance between the coupling nuclei. Attempts to obtain a ROESY spectra for this compound, which would have provided information on its conformation, were unsuccessful.

Fig. 5.11. ^1H-^1H COSY of Mequitamium depicting the region between 1.7 - 4.2 ppm. NMR conditions as described in section 2.4.4.



5.4.2. Complexation with α-, β- and γ-CD.

Table 5.16. gives the changes in mequitamium chemical shift (Δδ) observed upon complexation with the three parent CDs when analysed under equivalent conditions to those which produced optimum enantio-resolution using γ-CD in CE studies (section 4.4.).

Table 5.16. Chemical shift changes (Δδ) in ^{1}H-NMR spectra of mequitamium induced by various CDs. A negitive value indicates an upfield shift (shielded) whilst a positive one indicates a downfield shift (deshielded). Signal splitting patterns are the same as those given in Table 5.15. An asterisk indicates that the signal was obscured. NMR conditions: 10 mM CD, 9.5% MeCN-d_3-NaH_2Citrate (50 mM, pH 3.5). The analyte was added in an equimolar concentration.

Proton position	α-CD	β-CD	γ-CD
1,10/2,9/4,7	-0.002	-0.009	-0.031
3,8	+0.001	+0.008	-0.041
13a	-0.024	-0.020	-0.034
13b	*	-0.018	+0.039
14	-0.006	-0.007	-0.008
15[a]	-0.017, -0.015	-0.018, -0.014	-0.019, -0.013
16/20	-0.004	-0.018	+0.094
17	-0.009	-0.019	-0.047
18	-0.005	-0.007	-0.014
19	-0.008	-0.013	-0.033
21	-0.017	-0.018	-0.024

[a] These are the shifts observed for the two (AB) protons.

The magnitude of chemical shifts induced by α and b-CD are generally weaker than those observed in the presence of γ-CD. The pattern of shifts i.e. whether up

or downfield is identical for α- and β-CD but different in three instances to those induced by γ-CD. This difference in both shift patterns and strength may suggest that mequitamium binds with γ-CD in a dissimilar manner to that of α- and β-CD. α-CD produced the weakest shift changes especially in the alkyl proton signals. The cavity of α-CD may have been too small to accommodate the analyte's bulky non-aromatic ring, with the protons in positions 14, 18 , 17 and 19 showing little or no chemical shifts. Only the methyl group on carbon 21 and the methylene protons on carbon 15 showed any significant chemical shift (a shielding effect), perhaps from a partial inclusion by α-CD, which may have been sterically hindered from deeper inclusion by the nuclei in positions 19 and 20. The aromatic protons of mequitamium were not significantly affected in the presence of α-CD, suggesting that little or no host-guest interaction occurs in this region of the analyte.

β-CD produced slightly stronger chemical shifts than α-CD for the protons on 16/20, 17 and 19, whilst the shifts of 15 and 21 were very similar in magnitude to those caused by α-CD. The larger cavity dimensions of β-CD would allow it to envelop a bigger portion of the bridged alkyl ring structure, which could account for the observed difference in chemical shifts. Spatially, it would certainly be easier for β-CD to accommodate part of the tricyclic aromatic moiety into its cavity than the smaller α-CD and this seems to be reflected in the larger $\Delta\delta$ of all the aromatic protons when β-CD was present. Interestingly, the split triplet representing protons 3/8 was deshielded, whilst the remaining aromatic protons were then shifted upfield.

γ-CD produced the strongest $\Delta\delta$ in mequitamium although those shifts experienced by protons 14 and 15 did not significantly differ in the presence of any of the three parent CDs. The guest aromatic protons were moved strongly upfield, evidence of their inclusion by the macrocycle and subsequent shielding

from the externally applied magnetic field. Amato *et al* 1993 employed NMR and molecular modelling to study the interaction between certain CDs and suronacrine (9-[(phenylmethyl)amino]-1,2,3,4-tetrahydro-9-acridin-1-ol), which is structurally similar to mequitamum. They found that its phenyl ring entered the CD cavity from the secondary side so that its tricyclic moiety was brought into close contact with the opening of the cavity, especially in the case of β-CD. They discounted the molecules interaction with γ-CD as the analyte chemical shifts 'were not significant'. However, the more hydrophobic tricyclic moiety of mequitamium coupled with its bulkier alkyl substituient (not the planar phenyl ring of suronacrine), would seem to have enhanced the host-guest interaction, as supported by the obvious changes in mequitamium Δδ upon interaction with γ-CD. Furthermore, the increased size of the γ-CD cavity, 9.5 Å as compared to 7.8 Å of β-CD (Smith *et al* 1994), would allow the tricyclic group and hence the entire molecule, easier access to the inner CD cavity. γ-CD is capable of complexing with large bulky analytes such as fullerene-60 (Andersson *et al* 1994) and macrocyclic lanthanides (Sherry *et al* 1994). Therefore it could readily incorporate mequitamium within its cavity, explaining the observed shielding effects in both the tricyclic and alkyl moieties of the guest.

The protons on the mequitamium alkyl group experienced upfield Δδ's except for those of the 16/20 multiplet. They were moved very strongly downfield as was proton 13b of the inter-connecting methylene group. Guest Δδ changes in the presence of CDs may be caused by conformational changes induced by binding to a CD. The strong deshielding effect experienced by proton 13b when γ-CD was added could have been brought about by a conformational change in which this proton experienced a stronger deshielding effect of the nearby nitrogen nuclei. No deshielding was noted for this proton in the presence of any other CD. A similar deshielding effect occured for protons 16/20, which may have been caused by the connecting quaternary nitrogen nuclei exerting a powerful Δδ on these protons as

their spatial arrangements altered following CD complexation. Yamashoji *et al.* 1990 noted just such an effect when examining DL-alanine β-naphthylamide salts interacting with various CDs, where conformational changes of the guest molecule upon complexation with β-CD caused certain of the guest proton signals to experience a greater deshielding effect when electronegitive nuclei were nearby.

Perhaps the most notable aspect of the spectra in all three cases is the total lack of guest signal splitting, a factor which was expected at least in the presence of γ-CD, due to its previous ability to chirally resolve mequitamium by HPLC (section 3.4.) and CE (section 4.4.).

5.4.3. Chemical Shift of α-, β- and γ-CD Protons Upon Guest Interaction.

Table 5.17. shows the chemical shift values observed for the host CD protons when mequitamum was present (measured under those same conditions as the spectra in section 5.4.2.).

Table 5.17. Chemical Shift (Δδ) of α-, β- and γ-CD protons. A negative value indicates an upfield shift (shielded) whilst a positive one indicates a downfield shift (deshielded).

Cyclodextrin	H-1	H-2	H-3	H-4	H-5	6-CH_2
α-CD	-0.003	0.001	-0.004	-0.001	-0.003[a]	-0.003[a]
β-CD	-0.003	-0.004	-0.008	0.001	-0.007	-0.005
γ-CD	-0.004	-0.004	-0.015	-0.002	-0.013	-0.008

[a] It was not possible to differentiate between these two sets of signals due to spectral overlap.

The external protons of γ-CD i.e. H1, H2 and H4 were only slightly shifted compared to H3 and H5 when mequitamium was introduced. These latter mentioned protons are inside the CD cavity and clearly experienced an upfield shift (negative value) consistent with the insertion of an aromatic guest molecule (or an aromatic portion of a guest structure). β-CD underwent a similar though weaker affect to γ-CD while α-CD displayed rather smaller chemical shift changes which were not significantly different between the inner and outer CD proton positions.

5.4.4. Complexation with Hydroxypropyl Derivatized CDs.

The hydroxypropyl derivatives of α-, β- and γ-CD were examined to see if they would enhance the chiral recognition for mequitamium over that observed with the three parent CDs. The induced chemical shift changes of mequitamium (under the same conditions as stated for Table 5.16.) are shown in Table 5.18.(see p.175). No analyte signal splitting was observed with any of the CD derivatives.

The weakest analyte shifts were generally associated with the addition of HP-α-CD although in two instances, nuclei 15 and 18, HP-γ-CD induced no observed change in the mequitamium protons. HP-β-CD produced the greatest analyte Δδ overall, most notably in the case of the aromatic protons, 13a and 16/20. These suggest a greater interaction between HP-β-CD and mequitamium in these areas. The pattern of guest alkyl shifts i.e. up or downfield, was quite different in the case of each CD derivative e.g. the methyl group on carbon 21 was deshielded (+0.018 ppm) by HP-α-CD but shielded (-0.017 ppm) to an equal extent by HP-β-CD and HP-γ-CD, whilst proton 18 was deshielded by HP-α-CD, shielded by HP-β-CD but apparently not shifted by HP-γ-CD.

The deshielding of the methyl group on carbon 21 by HP-α-CD may, as has previously been stated, have been caused by a conformational change of the analyte induced by binding to the CD, where the methyl protons experience a greater deshielding effect from the adjoining quaternary nitrogen nuclei. Ion-dipole interactions could also have affected the δ of protons near the quaternary nitrogen nuclei.

Table 5.18. Mequitamium Δδ in the presence of HP-α-CD, HP-β-CD and HP-γ-CD. NMR conditions as stated in text. * Indicates the signal was obscured.

Proton Position	HP-α-CD	HP-β-CD	HP-γ-CD
1,10/2,9/4,7	+0.001	-0.013	-0.004
3,8	+0.004	+0.015	+0.006
13a	-0.007	-0.026	-0.014
13b	-0.009	-0.015	+0.011
14	-0.005	-0.005	+0.003
15	-0.011, -0.009	-0.012, -0.010	0
16/20	*	+0.031	+0.009
17	-0.009	-0.022	-0.038
18	+0.007	-0.006	0
19	+0.008	-0.017	-0.029
21	+0.018	-0.017	-0.017

Although the extended chains of these three CD derivatives also provide hydrogen-bonding sites that could aid chiral resolution, mequitamium itself does not possess any hydrogen nuclei which are directly capable of participating in such a bonding mechanism. Only the tertiary nitrogen nuclei could possibly act as hydrogen-bond acceptors with the HP chains if they were in the correct spatial orientation to do so. If however, as has been argued, this portion of mequitamium were to enter the CD cavity, it would not then be able to undergo hydrogen-bond formation with the external HP chains. Therefore, for this compound, any nega-

tion or enhancement of chiral resolution found with these derivatives using other analytical techniques (i.e. HPLC or FSCE) may not be attributable to their extended hydrogen-bonding capability.

Table 5.19. shows the proton shifts of the HP CD derivatives themselves, upon their interaction with mequitamium, which produced the largest shielding effect on the internal H3" and H5" protons of HP-β-CD, providing further proof of its inclusion complexation with this CD derivative. HP-γ-CD also displayed significant shielding of its internal protons, whilst the chemical shifts of HP-α-CD were generally lower and did not differ greatly between the inner and outer protons. Overall it would seem that the strength of host-guest interaction, as judged by analyte and CD chemical shift differences, is in the order HP-β-CD > HP-γ-CD > HP-α-CD.

Table 5.19. Chemical Shift (Δδ) data for the HP CD protons. NMR conditions as stated in Table 5.16.

CD type	1	2	3	4	5	6	HP side chains
HP-α-CD	+0.001	-0.002	-0.003	*	-0.004	-0.005	-0.007[a]
HP-β-CD	-0.006	-0.005	-0.009	*	-0.011	-0.006	-0.011[a]
HP-γ-CD	-0.002	-0.007	-0.007	*	-0.009	*	-0.008[a]

* Signal was obscured. [a] Average shift of the HP side chains.

There was not a great difference in the HP side chain chemical shifts observed for each CD derivative. This may again reflect the peripheral nature of their influence on the chiral resolution of this analyte, as previously remarked.

5.4.5. Complexation with Me and HE-β-CD.

Table 5.20. gives the chemical shifts for mequitamium in the presence of Me- and HE-β-CD under the same conditions as stated for Table 5.16. There is no significant difference between the chemical shifts of mequitamium with each CD. Also, no analyte signal splitting was observed. In view of these facts, any interaction between the analyte and either of the CDs would appear to have been rather weak.

Table 5. 20. Mequitamium Δδ in the presence of Me- and HE-β-CD. NMR conditions as stated for Table 5.16. * Signal obscured.

Analyte Nuclei Position (see Fig. 5.6)	Me-β-CD	HE-β-CD
1,10/2,9/4,7	+0.002	+0.005
3,8	+0.003	-0.003
13a	-0.009	-0.007
13b	-0.006	*
14	-0.005	+0.006
15	-0.011, -0.008	-0.007, -0.005
16/20	+0.013	+0.012
17	-0.008	-0.004
18	+0.010	+0.009
19	+0.005	+0.003
21	-0.010	-0.011

Table 5.21. on p.178 shows the measured shifts of Me- and HE-β-CD themselves when mequitamium was present. The inner protons, H3" and H5", do not show clear evidence of mequitamium inclusion. Their weak chemical shifts do not differ greatly from that of the outer CD protons. The Me and HE chain signals are

shifted more strongly than any of the other protons on their respective CDs. Perhaps they participated in a more external interaction with mequitamium i.e. one that involved the CD cavity less and hence was unable to provide for enantio-discrimination.

Table 5.21. Chemical Shift (Δδ) data for Me- and HE-β-CD protons.

NMR conditions as stated for Table 5.16. * Signal obscured.

CD type	1	2	3	4	5	6	Me-2	Me-3	He
Me-β-CD	-0.004	+0.002	-0.005	*	-0.004	+0.002	-0.010	-0.008	-
HE-β-CD	-0.001	-0.006	-0.004	*	-0.001	+0.004	-	-	-0.012

5.4.6 Calculation of Stoichiometery and Association Constant (K_f).

Using the procedure outlined in section 5.1.5. a Job (continuous variation) plot was used to determine the stoichiometry of each of the complexes formed between mequitamium and γ-CD and mequitamium and HP-β-CD. γ-CD and HP-β-CD had produced the greatest overall chemical shift data for mequtiamium when the host and guest were present in a 1:1 ratio and it was of interest see if the host:guest stoichiometry altered in the presence of the two different CDs. In each case four solutions of 9.5% acetonitrile-d_3 - NaH_2 Citrate (50 mM, pH 3.5) with varying molar ratios (mequitamium:CD) were prepared. The data for mequitamium:γ-CD and mequitamium:HP-β-CD complexes are presented in Tables 5.22. and 5.23. respectively (see p.179) and the actual plots are shown in Figure 5.12. (see p.180).

Table 5.22. Job Plot Data for 3/8 Protons of Mequitamium (Meq) : γ-CD (CD) Complex. NMR conditions as in Table 5.16. except for analyte:CD ratio.

[CD]	[Meq]	Δδ ppm	δHz	$\frac{\delta_{obs}Hz \times [CD]}{[CD] + [Meq]}$	$\frac{[Meq]}{[CD] + [Meq]}$
3/4	1/4	-0.0135	5.4	4.05	1/4
2/3	1/3	-0.0203	8.1	5.35	1/3
1/2	1/2	-0.0405	16.2	8.10	1/2
1/3	2/3	-0.0540	21.6	7.13	2/3
1/4	3/4	-0.0608	24.3	6.08	3/4

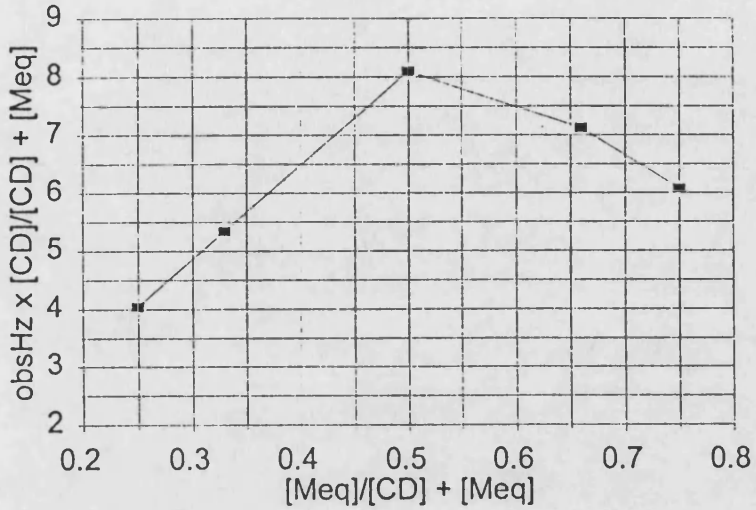
Table 5.23. Job Plot Data for 3/8 Protons of Mequitamium (Meq):HP-β-CD (CD) Complex. NMR conditions as stated in Table 5.22.

[CD]	[Meq]	Δδ ppm	δHz	$\frac{\delta_{obs}Hz \times [CD]}{[CD] + [Meq]}$	$\frac{[Meq]}{[CD] + [Meq]}$
3/4	1/4	+0.006	2.4	1.8	1/4
2/3	1/3	+0.010	4.0	2.64	1/3
1/2	1/2	+0.015	6.0	3.0	1/2
1/3	2/3	+0.017	6.8	2.24	2/3
1/4	3/4	+0.019	7.6	1.9	3/4

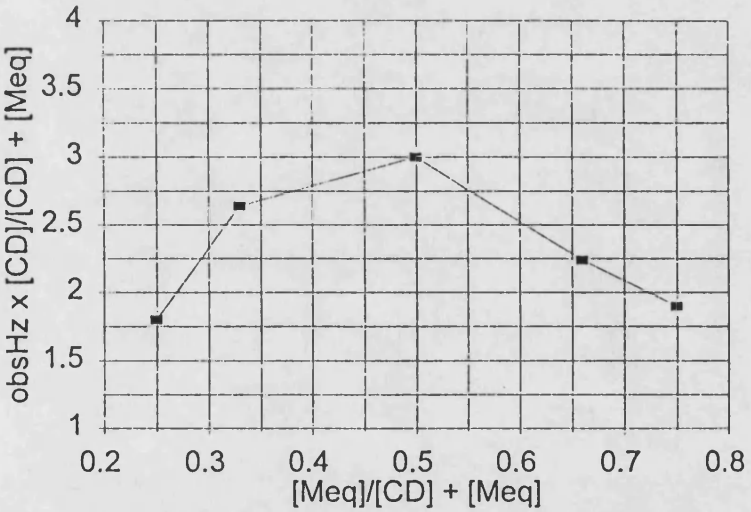
Each of the Job plots with γ-CD and HP-β-CD show a clear maximum at a [CD]/[CD] + [A] ratio of 0.5 indicating the formation of a 1:1 host:guest complex. With the complex stoichiometery being equal in the presence of these two CDs, there is no reason to suppose that any of the other CDs employed would form a mequitamium complex with a different stoichiometry.

Figure 5.12. Job Plots of (a) Mequitamium:γ-CD and (b) Mequitamium:HP-β-CD. Plots completed using chemical shifts of the mequitamium 3/8 protons signal. NMR conditions as stated for Tables 5.22. and 5.23.

(a) Mequitamium:γ-CD.



(b) Mequitamium:HP-β-CD.



The complex formation constant, K_f, was also determined for mequitamium with γ-CD and HP-β-CD under the experimental conditions described in 2.4.3. Using the procedure outlined in section 5.1.3., the chemical shift of one species (mequitamium) is measured in the excess of another species (a CD) and plotted by

the Foster-Fyfe method ($\Delta\delta_{obs}$ Hz/[CD] *versus* $\Delta\delta_{obs}$). The negitive slope of such plots affords the K_f value. Tables 5.24. and 5.25. detail the NMR data obtained form the experiments with γ-CD and HP-β-CD respectively, whilst Figure 5.13. on p. 182 shows the associated Foster-Fyfe plots of each mequitamium:CD complex.

Table 5.24. Association Constant, K_f, for Mequitamium:γ-CD complex. NMR conditions as stated in Table 5.16. except for analyte:CD concentrations. Data obtained using measured shifts of mequitamium 18 proton.

[Meq] mM	[γ-CD] mM	$\Delta\delta_{obs}$ Hz	Δδppm	ΔδHz/ [γ-CD]
3	5	5.4	-0.0135	1080
3	10	6.4	-0.0160	640
3	14	6.9	-0.0173	493
3	25	7.6	-0.0190	304

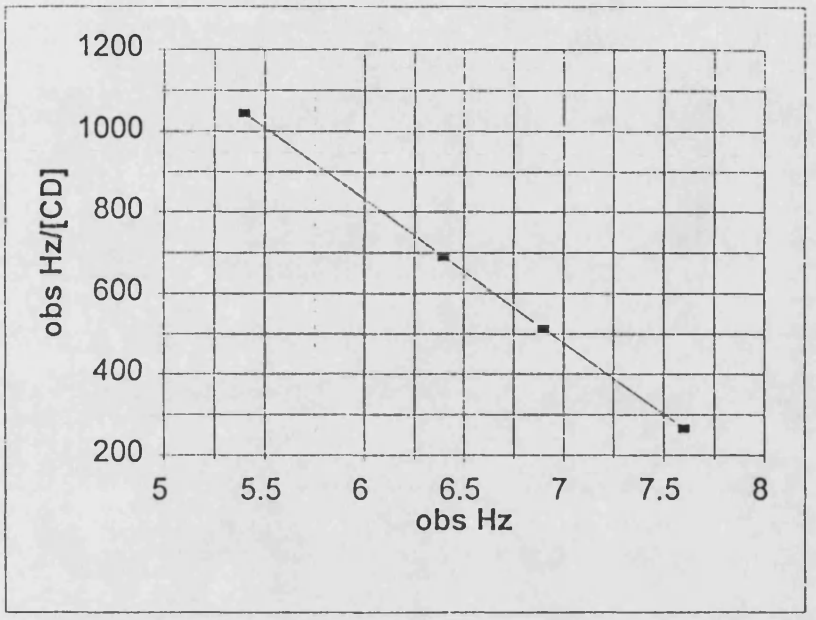
Table 5.25. Association Constant, K_f, for Mequitamium:HP-β-CD complex. NMR conditions as stated in Table 5.16 except for analyte:CD concentrations. Data obtained using measured shifts of mequitamium 13a proton.

[Meq] mM	[HP-β-CD] mM	$\Delta\delta_{obs}$ Hz	Δδppm	$\Delta\delta_{obs}$Hz/[HP-β-CD]
3	5	11.2	-0.028	2240
3	10	16.3	-0.041	1630
3	14	19.1	-0.048	1364
3	25	24.3	-0.061	972

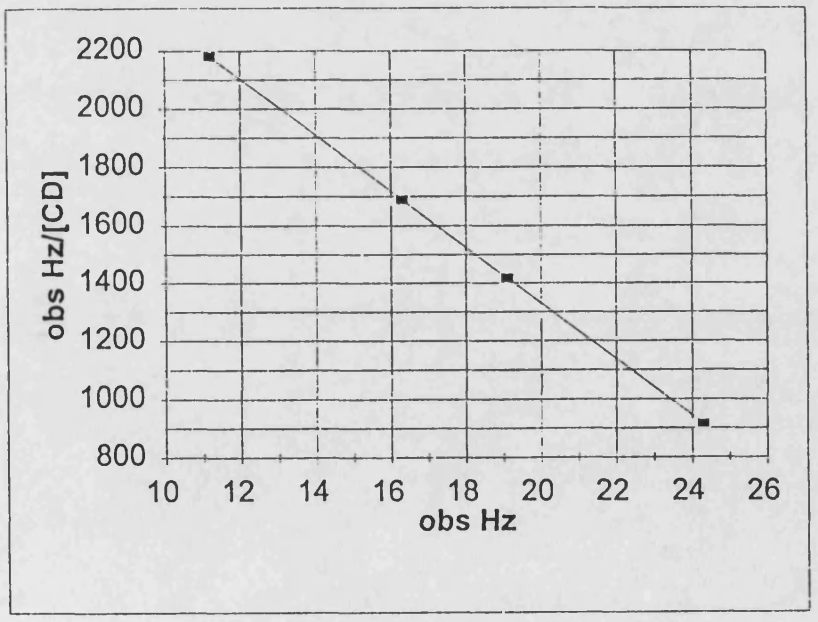
The K_f values calculated were 333 M^{-1} for mequitamium:γ-CD and 110 M^{-1} for mequitamium:HP-β-CD, indicating that γ-CD forms a stronger complex with mequitamium.

Figure 5.13. Foster-Fyfe plots of (a) mequitamium:γ-CD and (b) mequitamium:HP-β-CD. NMR data is given in Tables 5.24. and 5.25. respectively.

(a) Mequitamium:γ-CD



(b) Mequitamium:HP-β-CD.



5.4.7. Comparison of Results.

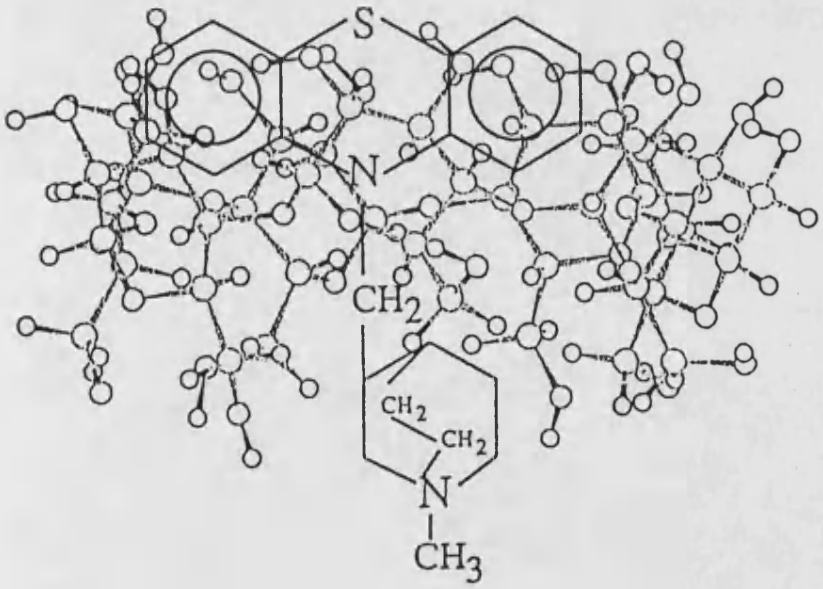
The greatest mequitamium chemical shift values (1:1 host-guest ratio) were recorded in the presence of γ-CD. The guest aromatic protons were especially strongly shielded indicating that inclusion complexation was significant. β-CD and α-CD induced smaller analyte chemical shifts than γ-CD and they also experienced weaker shielding of their H3" and H5" protons. These facts point to a weaker interaction of α-CD and β-CD with mequitamium probably because of the decreasing size of their cavities.

It is proposed that mequitamium enters into the cavity of γ-CD, accounting for both the upfield proton shifts of the guest (via a shielding effect) and certain guest downfield chemical shifts (due to conformational changes), which would also explain the strong shielding effect experienced by the inner protons of γ-CD itself. Due to the large size of the mequitamium structure, it seems prudent to suggest that inclusion interaction initiates at the wider secondary opening of γ-CD and not at the narrower primary end of the torus where steric constraints would prevent the large tricyclic moiety of mequitamium from entering the cavity. The difference between the H1"/H2" and 6-CH_2 proton shifts of β- and γ-CD (located at opposite ends of the CD torus) may have been caused by the bulky alkyl moiety of mequitamium protruding from the cavity at the primary end following complexation. This could have allowed the quaternary nitrogen of this group to influence the chemical environment around the 6-CH_2 protons via electronic interactions and so account for their observed chemical shift. Figure 5.14. on p.184 shows a schematic representation of the proposed inclusion complexation structure formed between γ-CD and mequitamium.

Chemical shifts were much more varied with the derivatised CDs than with the parent CDs. HP-γ-CD induced smaller analyte chemical shifts than its parent, γ-

CD, and showed less shielding of the inner H3" and H5" protons than γ-CD. This suggests a weaker association of HP-γ-CD with mequitamium, where derivatization of the normal CD hydroxy groups to form HP substituents, has reduced the ability of the macrocycle to affect enantio-resolution. Similarly, HP-α-CD did not increase guest chemical shifts over those observed with the parent α-CD. However HP-β-CD did produce larger guest chemical shifts than β-CD (especially of the aromatic protons). Increasing the number of derivatised groups around the rim of a CD can alter the size of the CD cavity due to a reduction of intramolecular hydrogen-bonding in the (OH)2 and (OH)3 positions (Ventura *et al.* 1994 and Inoue *et al.* 1987). This may have allowed mequitamium to interact more intimately with HP-β-CD than with the other two HP CD derivatives as a change in its cavity dimensions could have been more favourable for effective complexation with the guest molecule.

Figure 5.14. Schematic of Inclusion Complex formed between γ-CD and Mequitamium.



Comparing the host and guest chemical shifts obtained in the presence of the three HP CD derivatives with those found using the three parent CDs, an increasing interaction in the order γ-CD> HP-β-CD > HP-γ-CD > β-CD > α-CD ≃ HP-α-CD was found.

Me-β-CD and HE-β-CD did not produce any evidence of their having a significant interaction with mequitamium. Both the guest and host chemical shifts were smaller than those measured in the presence of the other CD types, except α- and HP-α-CD, where the chemical shifts covered an equally small range. Derivatization of the parent oligomers did not prove beneficial for enantio-resolution in these cases. The proposed inability of mequitamium to engage in hydrogen-bonding with the external hydroxy groups present on all of the CDs (except to an extent Me-β-CD) negates any possible improvement on enantio-resolution which these derivatives could incur through a hydrogen-bonding mechanism. Only subtle changes in cavity dimensions, as a consequence of certain hydroxy group modification, may have enabled HP-β-CD and to a lesser degree HP-γ-CD to provide for chiral discriminination.

The complex stoichiometery with γ-CD and HP-β-CD was demonstrated to have been the same i.e. 1:1 host/guest complexation ratio. With a large structure such as mequitamium it is unlikely that more than one molecule could enter into the CD torous at a time, whilst the large inner CD cavity of e.g. γ-CD would be quite capable of accommodating the full bulk of mequitamium, removing the chance of two CDs complexing the structure at the same time.

The association constant, K_f, of γ-CD with mequitamium (333 M^{-1}) was found to be greater than that of HP-β-CD (110 M^{-1}). This was reflected in the larger guest and host chemical shift strengths seen in the presence of γ-CD.

CHAPTER SIX
CONCLUSIONS

The initial two sections of chapter six attempt to draw comparisons between firstly, the various HPLC methodologies used in this work and secondly the results of the FSCE experiments when compared to those of the HPLC techniques. The ability of each method to provide chiral resolution for the analytes is discussed along with the advantages and disadvantages of each. The final section draws together these findings in conjunction with NMR spectroscopy data, in order to consider how the results of each technique may be related.

6.1. Overall Comparison of HPLC Methods for Chiral Enantio-Resolution.

All the analytes tested in this work have been examined using the following range of HPLC methods: bonded CD columns operated in the reversed phase mode, CDs as mobile phase additives in the reversed phase mode, bonded CD columns operated in the polar organic mode and CDs as mobile phase additives using porous graphitic carbon as a stationary phase. There are no publications which examine a similar range of HPLC methods to that employed here in order to draw comparisons between those enantio-resolutions obtained with different groups of chiral compounds. The following sections discuss the ability of the four stated HPLC methods to effect enantio-resolutions for the various analytes when examined in the presence of different CDs. The influence of analyte / CD structure on enantio-resolution is discussed, along with the role played by the relevant inherent chromatographic mechanisms.

6.1.1. Bonded CD column in Reversed Phase Mode.

All the analytes utilised i.e. the phenethylamines, propranolol analogues and mequitamium were examined using a β-CD column (250 x 4.6 mm) under a range of different reversed phase conditions (see Table 6.1. on p. 191 for the optimum results).

The CD molecules are tethered to 5 μm silica particles via an ether linkage on a carbon chain between 2 and 20 atoms in length (Technicol 1992). The point of attachment on the CD molecules is the primary hydroxyl groups. In order for enantio-separation to occur the analytes must form a stereospecific inclusion complex with the chiral selector, where the transient diastereoisomeric complexes have different stability constants such that they provide for chromatographic separation of the enantiomers (Pirkle and Welch 1992).

Only three of the phenethylamines, ephedrine, oxilofrine and orciprenaline and two of the propranolol analogues (compounds **2** and **5**) showed any signs of chiral resolution (previously unreported) using reversed phase methodology with a β-CD column. However, upon lowering the temperature to 10°C, compounds **1** and **4** also showed signs of chiral discrimination. Lamparczyk *et al.* 1994 have shown how the presence of multiple interaction/retention mechanisms can lead to non-linear van't Hoff plots. van't Hoff plots of compounds **1-6** suggested that all the analytes, except the unresolved compounds **3** and **6**, interacted in a similar fashion with the CD macrocycles, by means of more than one mechanism. Mequitamium did not resolve with either a β- or γ-CD bonded phase and eluted from both columns with k′ values < 4 under a range of differing mobile phase conditions.

β-CD is known to be able to complex substituted phenyl and naphthyl rings (Abidi and Mounts 1994, Rizzi *et al.* 1994 and Piperaki *et al.* 1994). The substituents on the aromatic moieties of the propranolol analogues were clearly influential in their inclusion complexation. A bulky and non-polar *t*-butyl group on the alkyl chain of compounds **2** and **5** promoted their chiral resolution over that of their *i*-propyl counterparts, compounds **1** and **4**. When examined at the same temperature, the k′ values of compounds **2** and **5** were also larger than compounds **1** and **4** suggesting a stronger interaction with the immobilised β-CD macrocycles on the surface of the column. The

hydrophilic conditions prevalent in the mobile phase may have encouraged the most non-polar of the analytes i.e. compounds **2** and **5** to interact more strongly with the apolar cavity of the β-CD molecules, which in turn would help to explain their increased chiral resolution by promoting other analyte-CD interactions e.g. hydrogen-bonding, dipole-dipole interactions. It is the presence of a number of such differing forces which is known to lead to chromatographic chiral resolution based on the three-point interaction model proposed by Dagleish 1952 and advanced by others e.g. Gaskell and Crooks 1991.

Compound **2** displayed higher chiral resolution than compound **5** (R′ = phenyl) and possessed a physically larger aromatic group (compound **2** R′ = naphthyl) which supports the idea that analyte steric bulk can influence resolution by causing a 'tighter fit' between the CD cavity and the analyte. The more tightly retained the analyte the greater influence its stereochemical structure would have on its interaction within the chiral environment of the CD molecule.

With the phenethylamines the position of the hydroxyl substituents on the phenyl ring was found to greatly alter the observed chiral resolutions. The structures of ephedrine and oxilofrine differ only in that oxilofrine possesses a hydroxyl group in the 4' position, yet its enantio-resolution was nearly twice that of ephedrine (67% CRF compared to 36% CRF). Sherrod 1992 has stated that CDs possess large dipole moments which may play an important role in analyte complexation depending on the strength and direction of the dipole moment in the guest molecule. The position of the hydroxyl group on oxilofrine will alter its dipole moment compared to that of ephedrine. It is this difference in the two molecules electrostatic nature which appears responsible for enhancing the chiral resolution of oxilofrine over that of ephedrine. The position of the hydroxyl groups on the phenyl ring was also found to be important. The enantiomers of orciprenaline were separated (39% CRF) while those of isoprenaline were not. The two compounds differ only in the position of their aromatic substituents i.e. orciprenaline has a 3,5-diOH substitution pattern and isoprenaline has a 3,4-diOH pattern. The plane of analyte

inclusion can be influenced by the positioning of polar groups on the guest structure (Technicol 1992), which would likely account for the results obtained here. If the molecules entered the CD cavity at different angles their alkyl chains (containing hydroxyl and secondary amine functions) would also be positioned differently so that the potential for dipolar and hydrogen-bonding interactions will vary, accounting for the observed changes in their enantio-resolutions.

The absence of chiral resolution and low k′ values obtained with mequitamium when employing either a β- or γ-CD bonded column in the reversed phase mode would suggest that this analyte interacts weakly with these two macrocycles. However, subsequent experiments have shown that mequitamium does complex significantly with γ-CD in the free state (see sections 3.4.3., 4.4.3. and 5.4.2.) i.e. when it is not bound to a silica surface. Tethering the γ-CD molecules has clearly resulted in a reduction of their ability to stereo-specifically interact with the enantiomers of mequitamium, possibly through constraints on the CD orientation or guest interaction with the linking spacer arm between the CD and stationary phase surface. The possibility of these effects occurring has been proposed before (Stalcup *et al.* 1990). Furthermore, section 3.4.5. states that the difference in K_f values for the R and S enantiomers may not be high enough to permit their resolution and that the observed chiral separation in section 3.4.3. was strongly influenced by the high retention of the guest-host complexes ($k'_{a.cd}$) on an achiral stationary phase. In view of these facts it may not be unexpected that enantio-resolution was not found for mequitamium using a CD bonded stationary phase.

Overall 7 analytes out of a total of 16 were chirally resolved to some extent using a bonded CD column in the reversed phase mode. Inclusion was favoured by the more hydrophobic of the analytes in the case of the propranolol analogues while the electrostatic nature and substitution pattern of the included aromatic ring of the phenethylamines was seen to be alter their chiral separation.

Table 6.1. Summary of optimum enantio-resolution factors measured using HPLC methodologies. See Chapter three for details.

Analyte	CD Bonded Column - Reversed Phase		CD Bonded Column - Polar Organic		CDs as Mobile Phase Additive - Reversed Phase (phenethylamine values are with β-CD)		CDs as Mobile Phase Additive - PGC Column	
	Resolution %CRF	k′	Resolution %CRF	k′	Resolution	k′	Resolution %CRF	k′
Ephedrine	36	4.3,4.4	0	43	0	4.2	<5[e] / 45[f]	3.8, 4.0[e] / 4.0, 4.5[f]
Oxedrine	0	2.8	-	46	0	3.4	0	2.3
Oxilofrine	67	3.5,3.8	0	>100	0	3.3	35[e]/ 82[f]	2.8, 3.3[e] / 3.3, 3.8[f]
Norfenefrine	0	2.1	-	>100	0	2.4	0	2.1
Etilefrine	0	2.9	0	52	0	2.6	0	2.7
Orciprenaline	39	3.2,3.4	0	80	0	3.0	20 / 78[f]	1.8, 2.3[e] / 1.3, 2.3[f]
Noradrenaline	0	2.3	-	>100	0	2.1	0	2.2
Isoprenaline	0	2.9	0	90	0	2.2	0	3.1
Salbutamol	0	2.2	32	59,62	0	1.9	0	3.0
Propranolol (1)	9[d]	13.3, 13.4	62	16.8, 17.2	0	13.5 [a]	0	6.6
Compound 2	43	12.5,13.0	89	12.5, 12.8	30 %CRF[a]	24.1, 24.3 [a]	0	7.2
Compound 3	0	11.1	17	20.0, 20.3	0	11.4 [a]	0	5.4
Compound 4	7[d]	6.4, 6.5	78	17.9, 18.3	0	3.2 [a]	42[b]	0.8, 0.9
Compound 5	32	7.1, 7.6	93	13.3, 13.6	0	4.1 [a]	47[b]	0.9, 1.0
Compound 6	0	20.2	0	<3	0	52 [a]	0	11.8
Mequitamium	0	<4	0	<2	1.06 R_s[c] / 0.3 R_s[g]	18.7, 20.7 [c] / 6.75, 7.74 [g]	0	>100

[a] Obtained with 12 mM HE-β-CD. [b] Obtained with 2.5 mM Peracetyl-β-CD. [c] Obtained with 12 mM γ-CD. [d] Measured at 10°C. [e] Obtained with 2.5 mM β-CD. [f] Obtained with 2.5 mM peracetyl-β-CD.

[g] Obtained with 12 mM HP-β-CD. - Not measured due to long analyte retention on column.

6.1.2. Bonded CD Column in Polar Organic Mode.

The use of polar organic mobile phases is a relatively recent approach for chiral separations in HPLC. Armstrong *et al.* 1992 and Zukowski *et al.* 1992 were amongst the first to publish work which tentatively dealt with the subject. Since then there has been much debate on the exact nature of the operating mechanism which is responsible for the observed chiral resolutions (see section 3.1.3. for a more details). Although all the phenethylamines possess more than one hydrogen-bond donor/acceptor group, which is believed to be a necessary prerequisite for chiral separation in the polar organic mode (Technicol 1992, Chang *et al.* 1993), only one phenethylamine, salbutamol, was chirally resolved (32% CRF, k′ = 59, 62) compared to three other phenethylamines in the CD bonded reversed phase mode. Sterically, salbutamol is the most bulky of the phenethylamines and it is more non-polar than the others, containing a *t*-butyl group on its alkyl chain. All the phenethylamines had much larger k′ values on the β-CD bonded column in the polar organic mode (all k′ values >40, see section 3.2.2.) than when using the same column under reversed phase conditions (all k′ values <5, see section 3.2.1.). The solubility of the analytes in the high organic content mobile phase may have been reduced, which would have contributed to these long k′ values. The selectivity of the β-CD molecules and therefore their interaction with the guest analytes was clearly different in the two HPLC modes.

This view is supported by an examination of the results obtained with the propranolol analogues. As explained in section 3.3.5. the order of analyte retention was unique in the polar organic mode (compound **3>4>1>5>2>6**) and the relative strength of analyte chiral resolutions (compound **2 > 5, 1> 4** and **3 = 6**) was also different in every case to those obtained in the reversed phase CD bonded mode, with compound **5 > 2, 4 >1** and **3 >6**. Increasing the hydrophobicity of the propranolol analytes caused a decrease in k′ values but an increase in chiral separations. It is proposed here that a size dependant repulsive steric effect was responsible for this observed trend in chiral resolutions (Matchett *et al.* 1996).The effects of structural

alterations in the non-polar regions of the analytes were found to exert profound changes upon chiral resolution and capacity values, indicating that features which cannot hydrogen-bond with the CD molecule can still play an important role in this chiral recognition process. This mechanism agrees with the proposal that analytes do not enter the CD cavity in the polar organic mode due to the high percentage of organic modifier (Armstrong *et al.* 1992 and Chang *et al.* 1992) but rather come into closer contact with the external structure of the macrocycle e.g. positioning themselves as a 'lid' over the CD cavity. The inability of compound **6** to display chiral resolution and its rapid elution from the column was linked to the urea linkage in its alkyl chain, which reduced or negated the ability of the molecule to engage in hydrogen-bonding with the external CD hydroxyl groups.

Those three compounds which showed the largest enantio-resolutions from their respective analyte groups i.e. the phenethylamine salbutamol and the propranolol analogues, compounds **2** and **5**, all possessed a *t*-butyl group on their alkyl chains. These alkyl chains were identical except that compounds **2** and **5** possessed an extra -0-CH2- link at the beginning. The *t*-butyl group would seem then to have been important in the successful chiral resolutions of both classes of compounds, which means that the alkyl chains of the analytes must be in relatively close contact with the CD molecules. The greatest structural difference in the three analytes lay in their aromatic moieties. The phenyl and naphthyl rings of compounds **5** and **2** respectively allowed for greater chiral separation than the 3,4-substituted ring of salbutamol, which appears to have reduced the ability of salbutamol to stereospecifically interact with the β-CD molecules.

6.1.3. CDs as Mobile Phase Additives - Reversed Phase Mode.

It has previously been established that chiral resolution in this mode may occur as a result of the differences in the adsorption of analyte-CD complexes on the stationary phase and /or differences in the K_f values of the complexes themselves (Sybilska *et*

al. 1986 and Zukowski *et al.* 1988). Section 3.1.5. details the equilibria involved in CD modified HPLC systems. This approach is more flexible than the two previously mentioned HPLC methods in that different mobile phases containing various CDs can be quickly prepared and equilibrated on different achiral stationary phases. One additional factor which may be varied here is the actual concentration of the CD additive although this is clearly limited by its solubility in the mobile phase. Also the achiral stationary phase itself can readily be changed.

Despite the potential to alter more variables in the chromatographic process this was the least successful of the HPLC methods to be tested. None of the phenethylamines could be chirally separated despite being examined in the presence of over seven different CD types dissolved in the mobile phase (see section 3.2.3.). There are no reports in the literature concerning the successful chiral resolution of any of the phenethylamines examined in this work when using CDs as mobile phase additives without prior derivatisation of the analytes. Mularz *et al.* 1988 were however able to resolve the enantiomers of pseudoephedrine, which is the diastereoisomer of ephedrine. They proposed, via chromatographic and NMR analysis, that the inability of β-CD as an additive to resolve the enantiomers of ephedrine was caused by the arrangement of the analytes amine and hydroxyl functions in relation to the hydroxyl groups of the CD.

Many of the phenethylamines examined here showed changes in their k′ values in the presence of the different CDs, indicating that they may have formed inclusion complexes. The absence of any observed enantio-specific resolution in these HPLC experiments would suggest that none of the phenethylamines formed stereospecific inclusion complexes with any of the tested CDs. However further analysis using FSCE and NMR (see sections 4.2.1. and 5.2.2. respectively) has shown that many of these analytes may be chirally resolved with several of those CDs unsuccessfully employed as mobile phase additives. One possible reason for the absence of observable enantio-resolution when using this form of HPLC is the generally low chromatographic efficiency of the system. Resolution is strongly influenced by the

number of theoretical plates and the addition of CDs to a mobile phase has been shown to lower column efficiency by as much as 30% (Sybilska *et al.* 1992). The measured efficiencies in these experiments were between 8,000 and 12,000 theoretical plates, which is not very high for a reversed phase column. If the CDs were responsible for lowering the column efficiency this would also reduce the potential to observe chiral resolution and may explain the absence of such a phenomenon in these experiments. FSCE showed that chiral resolution could be obtained with β-CD, one of those CDs tested as a mobile phase additive in the HPLC experiments, which then provided no resolution. The very high column efficiencies present in FSCE, averaging > 200,000 plates in this work, may have been the key element in determining that enantio-resolution was actually occurring.

The only analyte from amongst the six propranolol analogues to be chirally resolved was compound **2** (R′ = naphthyl, R = *t*-butyl) using HE-β-CD as the chiral additive to give a CRF value of 30%. Despite changes to the chromatographic conditions e.g. pH, organic modifier, CD concentration, no other instances of chiral resolution were recorded. All of the CDs tested in section 3.2.3. reduced the k′ values of the propranolol analogues when compared to the k′ values of the uncomplexed analytes, except for α-CD which produced little change. As in the case of the phenethylamines, this suggests that the propranolol analogues were complexing with the CDs, though not stereo-specifically. However, enantio-resolution in FSCE (sections 4.3.1. to 4.3.4.) and signal splitting in NMR studies (sections 5.3.1. and 5.3.3.) have established that chiral complexation can occur with several of the CDs unsuccessfully tested in this HPLC mode.

Mularz 1988 has reported the separation of the enantiomers of propranolol (α = 1.04) using β-CD as a mobile phase additive in conjunction with a reversed phase column. However no separation of propranolol could be achieved here under similar chromatographic conditions. Besides the obvious importance of the CD type, the nature of the stationary phase can also be influential for chiral resolution. Takeuchi and Nagae 1992, employing γ-CD as a mobile phase additive, have shown that the

nature of the stationary phase may affect the ability of the system to provide chiral separation. Walhagen and Edholm 1991 have also demonstrated how different commercially available stationary phases with ostensibly equivalent packings i.e. C18, could dramatically effect the chiral resolution of terbutaline enantiomers using β-CD as a mobile phase additive. These findings may help to account for the inability to achieve analyte chiral resolution using β-CD in this work, where a C8 Inertsil column was employed in comparison to a C18 column used by Mularz 1988. Guest enantiomers may interact with the chiral selector via a two point interaction and then contact simultaneously a solid surface, the stationary phase, so that the transient diastereomeric complexes are retained to different extents, which will then lead to their chiral resolution (Davankov and Kurganov 1983). Stationary phases with different extents of surface coverage and / or different carbon chain lengths are likely to show different selectivities which would help account here for the observed lack of chiral resolution of propranolol. The reduction in column efficiency noted when using CDs as mobile phase additives may also have been a contributing factor (Sybilska *et al.* 1992).

Mequitamium was the only analyte examined which was well resolved using this HPLC method. γ-CD and HP-β-CD were able to resolve the enantiomers of mequitamium, giving R_s values of 1.06 and 0.3 respectively (section 3.4.3.). Because of the high retention of mequitamium on a conventional 250 x 4.6 mm reversed phase column, two pKb pre-columns (20 x 4.6 mm) were used as the stationary phase in order to lower k′ values within practical limits. However, this system had a very low chromatographic efficiency, 2500 - 4500 plates, which reduced the R_s of the enantiomers despite their measured selectivity (α) value of 1.11. Higher resolution values were obtained in FSCE (section 4.4.1.) using γ-CD (R_s = 1.82), HP-β-CD (R_s = 1.22) and also HP-γ-CD (R_s = 0.86). The latter mentioned CD did not display any resolution of mequitamium's enantiomers in this HPLC mode. The high efficiency of the FSCE systems with > 200,000 plates would clearly have helped in the achievement of these greater R_s values.

One interesting feature of the changes in the k′ values of mequitamium was that β-CD and HP-γ-CD reduced them to a level below that caused by γ-CD and HP-β-CD, perhaps indicating stronger inclusion complexation, but they did not produce enantio-resolution. As stated above however, HP-γ-CD did then demonstrate chiral resolution of mequitamium in FSCE (section 4.4.1.) although β-CD still did not. Also, NMR studies (section 5.4.2. and 5.4.4.) showed that HP-γ-CD induced stronger chemical shifts of mequitamium than β-CD, though neither CD caused signal splitting. Therefore although β-CD can form inclusion complexes with mequitamium there is no evidence for such complexes being stereo-specific unlike those formed with HP-γ-CD.

It was also found that the nature of the organic modifier affected the resolution of mequitamium. MeOH removed observable chiral separation when used in place of MeCN at the same concentration. Li and Purdy 1992 found that different organic modifiers could generate different selectivities when using a bonded CD column. It is not unexpected then that a similar effect could occur when using CDs as mobile phase additives. According to Sherrod 1992, solvation can radically alter both the geometry and the relative stabilities of various conformations, which would help to explain the differences found in the abilites of the two organic modifiers to induce chiral resolution.

When the K_f values for each enantiomer were calculated in the presence of varying amounts of γ-CD (see section 3.4.3.) there was little difference between the values (R K_f= 396 M^{-1}, S K_f = 427 M^{-1}). Therefore the calculated $k'_{a.cd}$ values of each enantiomer-CD complex (R $k'_{a.cd}$ = 8.5, S $k'_{a.cd}$ = 6.8) seem to have had a stronger influence on their measured chiral resolution. Sybilska *et al.* 1986 have also shown how chiral resolution may arise solely as a result of differential adsorption of the diastereomeric complexes of mephenytoin on a C18 column. The separation of the enantiomers of mequitamium appears to be another example of chiral resolution occurring by the same mechanism proposed by Sybilska *et al.* 1986.

6.1.4. CDs as Mobile Phase Additives - PGC Column.

The unique surface of porous graphitic carbon (PGC) may be expected to provide different selectivities to that of standard reversed phase columns such as C18 and C8. The 2 dimensional surface with no functional groups, provides an extremely uniform phase where analyte retention is strongly influenced by electronic donor-acceptor interactions with the delocalised electron conduction bands of the PGC. Planar molecules have been shown to be preferentially retained by virtue of the flat surface of the column and high levels of organic modifiers are often required to elute analytes (Tanaka *et al.* 1993).

Three phenethylamines, ephedrine, oxilofrine and orciprenaline were chirally resolved using 2.5 mM β-CD as a mobile phase additive on the PGC column, where the organic modifier was 30% v/v. Chiral resolutions were lower than those achieved using a β-CD bonded column (section 3.2.1.) but higher than those observed using β-CD as a mobile phase additive with a C8 column (section 3.2.3.). The chiral separation of these three analytes was greatly improved when peracetyl-β-CD was used in place of β-CD (see Table 6.1. on p.190). Peracetyl-β-CD is insoluble in water and is also unable to hydrogen-bond donate from the 2, 3 or 6 positions, unlike any of the other CDs examined. Of the six propranolol analogues, compounds **4** and **5** (R′ = phenyl) were uniquely resolved in under two minutes using peracetyl-β-CD. None of the other analogues, which all have R′ = naphthyl, were separated. This may indicate that these larger analytes were hindered from interacting with the CD, perhaps by steric crowding as a result of the acetyl chains on the rim of peracetyl-β-CD preventing close interaction between the bulky analytes and the CD interior. Or, as postulated below, electron rich groups on the analytes e.g. naphthyl rings, may be preferentially interacting with the PGC surface. These previously unreported results demonstrate that correct selection of the stationary phase can be advantageous in chiral separations with CDs.

Mequitamium did not resolve in the presence of any of the CDs examined (section

3.4.4.). It displayed very high k′ values probably as a result of its electron rich tricyclic system interacting with the delocalised electron conduction bands of PGC (Bell *et al.* 1994). The high levels of organic modifier required (< 60%) to give practical elution times are likely to have reduced the ability of the analyte to interact with any of the CDs, which would help to account for the absence of chiral resolution.

Of all the HPLC methods examined, chiral resolution was greatest in the polar organic mode (6.1.2.), especially with the propranolol analogues. A unique size dependent, steric repulsive effect was proposed to explain the observed %CRF and k′ values. Resolution was generally very poor using CDs as mobile phase additives, either with standard reversed phase columns (section 6.1.1.) or a PGC column (6.1.4.). The low chromatographic efficiency of these methods was suggested as a major factor in their inability to show significant chiral resolution. A bonded CD column operated in the reversed phase mode (6.1.1.) proved able to resolve over half the analytes examined. However, immobilisation of β- and γ-CD onto a solid chromatographic support was found to engender different selectivities of the CDs towards the analytes when compared to the CDs as mobile phase additives.

6.2. Comparison of FSCE and HPLC Results.

One of the first comparative reports of HPLC and FSCE for chiral resolution was by Gassman *et al.* 1985 although they did not employ CDs as the chiral selector. They attempted to resolve the enantiomers of a number of dansyl amino acids using a FSCE buffer containing Cu (II) ions and L-histidine i.e. via a chiral ligand exchange mechanism. However the separation values obtained were not as good as those found using HPLC. In a non-chiral application, Terabe *et al.* 1988 observed that FSCE provided better separation of oxygen isotopic benzoic acids than HPLC, even though the number of theoretical plates was approximately 30,000 which is low for FSCE. There are few reports comparing the abilities of HPLC and FSCE to provide chiral

separations using CDs. Szemán and Ganzler 1994 used coated β- and γ-CD columns in FSCE for the separation of several compounds including hexobarbital and ephedrine. In the same work they used carboxymethyl- and carboxyethyl-β-CD as mobile phase additives in HPLC to again study the same compounds. However, because they did not employ the same chiral selectors in each analytical method a direct comparison of their results is impossible. In a comprehensive review of capillary electrophoresis, HPLC and GC, Issaq 1994 concluded that capillary electrophoresis provided higher efficiencies and better chiral separations for a large selection of chiral analytes than either of the other two methods.

Sections 3.1.1. - 3.1.6., 4.1.1. and 4.1.7. give details of the under-lying theories involved in the analysis of chiral analytes when CDs are utilised in various HPLC and FSCE methodologies. The following sections compare and contrast the chiral separations obtained with the analytes employed in this work when they were examined in the presence of different CDs using FSCE and HPLC.

6.2.1. Phenethylamine Analogues - Comparison of HPLC and FSCE Results.

Chiral resolution values were measured using %CRF (see equation 2.05) in the HPLC studies as enantiomer separations were generally not very high. In FSCE the parameter for measuring chiral separation was R_S (see equation 2.04), which is more usefully employed when peak separations are relatively large. A %CRF of 100% equals a R_S value of 1.5. R_S values higher than 1.5 cannot be measured using %CRF and consequently such peak separations are reported using the R_S function.

The highest chiral separations recorded for all of the phenethylamines were achieved using FSCE with Ac-β-CD as the chiral selector dissolved in the running buffer (see Table 6.2., p.200). Unfortunately it was not possible to use Ac-β-CD in HPLC as this CD derivative was especially prepared in the Pharmazeutisches Institut, Bonn and there was insufficient CD to prepared a mobile phase for HPLC work. Typically

around 500 ml of mobile phase was required to flush the HPLC system and to equilibrate the column. In the FSCE studies only 1 - 5 ml of CD containing buffer was required. This in itself is a major advantage of FSCE over HPLC: the ability to employ small amounts of chiral selectors which may be in short supply and yet still achieve chiral resolution.

β-CD was variously employed in HPLC and FSCE (see Table 6.2.). This CD resolved the enantiomers of oxilofrine and orciprenaline in both techniques, separated ephedrine and salbutamol enantiomers in HPLC only and resolved etilefrine by FSCE only. Only noradrenaline and isoprenaline were not separated by either FSCE or HPLC methods. The highest chiral resolution values of oxilofrine and orciprenaline were found using FSCE rather than HPLC (FSCE R_S of 1.16 compared to HPLC 67%CRF $\simeq$ 1.01 R_S for oxilofrine and FSCE R_S of 0.89 compared to HPLC 39%CRF $\simeq$ 0.59 R_S). Both these HPLC separations occurred using a bonded β-CD column. When β-CD was dissolved in an HPLC mobile phase in a similar approach to FSCE, chiral resolution was either non-existant or very low (Table 6.2.). If chiral resolution could be achieved using β-CD as a buffer additive in FSCE but not in HPLC, either the much lower efficiency of the HPLC column was responsible viz. chiral interaction was occurring but the efficiency of the system was too low to observe optical resolution, or stereo-specific interaction between the analytes and the β-CD molecules was in some way adversely affected by the stationary phase of the HPLC column. However, as the interaction of the phenethylamines on a C8 column in the absence of any CD was low (k' values were < 6) and the adsorption of β-CD onto reversed phase columns is also believed to be low (Zukowski *et al.* 1988), the influence of the stationary phase in this case may not have been strong. A more plausible reason then for the lack of chiral resolution is the low efficiency of the HPLC system (8000 - 12000 theoretical plates), which is exacerbated in the presence of CDs as stated in section 6.1.3.

Table 6.2. Comparison of phenethylamine resolution in HPLC and FSCE.

Analyte	%CRF by HPLC[a]			R_S in FSCE with β-CD	R_S in FSCE with Ac-β-CD
	RP-Bonded β-CD	PGC β-CD	PGC PerAc-β-CD		
ephedrine	36	<5	45	0	1.25
oxedrine	0	0	0	0	1.62
oxilofrine	67	35	35	1.16	1.74
norfenefrine	0	0	0	0	1.31
etilefrine	0	0	0	0.47	4.28
orciprenaline	39	20	78	0.89	1.32
noradrenaline	0	0	0	0	0
isoprenaline	0	0	0	0	0
salbutamol	32[b]	0	0	0	0.85

100% CRF equals R_S value of 1.5. [a] No chiral resolution was obtained using CDs as mobile phase additives in the reversed phase HPLC mode. [b] %CRF obtained in polar organic mode.

The unique separation of salbutamol enantiomers in the polar organic mode shows that the flexibilty of HPLC methodology can still provide an advantage over FSCE, where there are currently few reports of CDs bound to a column. Mayer and Schurig 1992, Armstrong *et al.* 1993 and Cruzado and Vigh 1992 have all used derivatised CDs bound to fused silica capillaries for separations in capillary electrophoresis but these phases are not readily available and several of them suffer from low stabilities and efficiencies.

Peracetyl-β-CD provided better chiral separation for ephedrine and orciprenaline enantiomers, in conjunction with a PGC column, than did any other method except FSCE with Ac-β-CD. Furthermore, it also resolved the enantiomers of oxilofrine (%CRF = 35%). It was not possible to employ this water insoluble CD derivative in FSCE buffers because the high levels of organic modifier necessary for its dissolution (> 35%) prevented the generation of a stable current. Again, this demonstrates some of the limiting factors in FSCE which do not apply in HPLC.

6.2.2. Propranolol Analogues - Comparison of HPLC and FSCE Results.

In HPLC experiments the propranolol analogues were best separated using a bonded β-CD column operated in the polar organic mode while in FSCE experiments HE-β-CD provided the highest R_S. Table 6.3. on page 204 shows the chiral resolution values obtained for the propranolol analogues using both analytical techniques.

Overall chiral resolution values for compounds **1-3** and compound **5** were highest when using FSCE, whilst the polar organic mode of HPLC provided the largest chiral separation for compound **4** (see Table 6.3.). Compound **6** was not resolved in HPLC and the weakly basic nature of its urea linkage prevented its examination by FSCE as the analyte could not be readily protonated, which resulted in impractical migration times, usually greater than one and a half hours.

It is of interest to note that HE-β-CD provided the highest enantio-resolution values for all the propranolol analogues in FSCE and that it was also the only CD to display any chiral separation, for compound **2**, when used as a mobile phase additive in HPLC. Furthermore, compound **2** had the largest R_S value of all the propranolol analogues in FSCE. There were also very strong reductions for all the analyte's k' values upon the addition of HE-β-CD to the mobile phase in HPLC (see Fig. 3.16. in section 3.3.3.), indicating that analyte-CD complexation, whether stereospecific or not, was certainly occurring. Therefore it may have been possible that chiral interaction between HE-β-CD and the other analogues did happen in HPLC. However, the efficiency of the HPLC system (10,000 - 12,000 theoretical plates) may have permitted only the strongest analyte:HE-β-CD interaction, that involving compound **2**, to become apparent as a chromatographic chiral resolution. β-CD and Me-β-CD were also more successfully employed as additives in FSCE than HPLC. This may again reflect on the low efficiency of the HPLC set-up as both CDs were also found to lower the k' value of compounds **1-5** upon their addition to the mobile phase indicating that complexation occurred.

Table 6.3. Comparison of enantio-resolution values for propranolol analogues in HPLC and FSCE.

Analyte	% CRF by HPLC[a]			R_S in FSCE			
	RP-Bonded β-CD	PGC PerAc-β-CD	Polar Organic[b]	β-CD	Ac-β-CD	HE-β-CD	Me-β-CD
Propranolol (1)	9	0	62 (0.83)	1.05	0.26	0.83	0.54
Compound 2	43	0	89 (1.34)	1.31	0.81	1.67	0.73
Compound 3	0	0	17 (0.26)	0.28	0	0.77	0.32
Compound 4	7	42	78 (1.17)	<0.1	0	1.03	0.68
Compound 5	32	47	93 (1.40)	0.63	0	1.54	1.54
Compound 6	0	0	0	-	-	-	-

[a] Only compound **2** was resolved (30%CRF) with HE-β-CD as mobile phase additive in reversed phase HPLC. [b] Comparative R_S values are given in parenthesis.

The ability of β-CD when employed in the polar organic mode to enable greatly improve analyte enantio-resolution values compared to those of the other HPLC techniques (see Table 6.3.) supports other evidence (section 6.1.2.) that this analyte-CD interaction mechanism is somewhat different to that present in the other HPLC and FSCE conditions.

Analyte migration times in FSCE were similar in length to chromatographic retention times except in the case of compounds **4** and **5**. When these two analytes, with R′ = phenyl, were examined with peracetyl-β-CD on a PGC column, k′ values were < 1.1 corresponding to analysis times of under one minute. As explained in section 6.1.4. the steric bulk and electronic nature of the analytes seems important in interactions with this CD on the PGC stationary phase. It is also possible that adsorption of the hydrophobic peracetyl-β-CD onto the PGC surface may have generated a 'dynamic chiral stationary phase' as postulated in section 3.4.4. Zukowski and Nowakowski 1989 and Zukowski *et al.* 1988 have reported the generation of such phases although only when using ODS columns and permethylated CDs. The very low capacity values

of compounds 4 and 5, coupled with their chiral resolution, would be readily explained by the adsorption of peracetyl-β-CD onto the PGC surface allowing chiral interaction with the analytes while hastening their elution from the column by reducing their direct interaction with the PGC column.

6.2.3. Mequitamium - Comparison of HPLC and FSCE Results.

Table 6.4. on page 205 shows the enantio-resolution values achieved for mequitamium in HPLC and FSCE. Of the ten different CDs variously examined (see sections 3.4. and 4.4.) only three provided chiral resolution and one of those, HP-γ-CD was effective only as a mobile phase additive in FSCE and not in HPLC.

Both the HPLC mobile phase and the FSCE running buffer were similar in make-up (aqueous solutions containing CDs and approximately 9 - 10 % MeCN) so they would not have been expected to play a major role in the chiral resolution differences observed with each technique and recorded in Table 6.4.

Table 6.4. Summary of maximum enantio-resolution values of mequitamium in HPLC and FSCE.

Analyte	R_S by HPLC[a] mobile phase additive			R_S in FSCE		
	γ-CD	HP-γ-CD	HP-β-CD	γ-CD	HP-γ-CD	HP-β-CD
Mequitamium	1.06	0	0.3	1.82	0.86	1.22

[a] No chiral resolution was obtained using any of the other HPLC methodologies.

It was noted in HPLC and FSCE that the nature of the buffer was crucial for chiral resolution of the mequitamium enantiomers. A KH_2PO_4 buffer prevented chiral recognition when used in place of NaH_2citrate, *ceteris paribus*. Increasing the buffer concentration from 50 to 75 or 100 mM lowered resolution of the mequitamium enantiomers while increasing migration times. Increased buffer levels reduce EOF

(Weinberger 1991) and are known to compete with analytes for the CD cavity (Technicol 1992).

Furthermore, when MeOH was substituted for MeCN, HPLC analysis times more than doubled and enantio-resolution was removed. As the percentage of MeCN was varied in FSCE the separation of the mequitamium enantiomers reached a maximum at 9.5 % MeCN. Migration times also increased with rising MeCN concentration, up to a maximum of over 16 minutes, also at 9.5% MeCN (see Table 4.9., p.127). Such enantio-selectivity differences in the presence of various buffer additives have been noted previously in HPLC. They are believed to occur as a result of competitive interaction between the analyte and the additive for the CD cavity (Technicol 1992) and/or the ability of organic modifiers to alter the geometry and relative stabilities of complexing species in solution (Sherrod 1992). Wren and Rowe 1992 have suggested that this effect may also occur in FSCE, with induced changes in chiral resolution of the analyte also being dependent upon the CD concentration. Resolution values will approach a maximum as the CD concentration is raised and then begin to fall above this optimum level. The results found in section 4.4. agree with these proposals and demonstrate that it is necessary to adjust a large number of analytical variables before definitive conclusions concerning the presence or absence of chiral resolution can be made.

Chiral resolution values of mequitamium were significantly higher in FSCE than in HPLC. As stated in section 6.1.3. this discrepancy between HPLC and FSCE analyte separation ability may be related to the HPLC system having only 2500 - 4500 theoretical plates, whilst the FSCE column routinely produced values > 200,000.

The K_f values for the enantiomers of mequitamium were measured in HPLC and FSCE by varying the concentration of γ-CD in solution. HPLC experiments gave $K_f R = 396$, $K_f S = 427\ M^{-1}$ and FSCE experiments gave $K_f R = 316$ and $K_f S = 355\ M^{-1}$ (see sections 3.4.3. and 4.4.4. respectively for further details). The results from the two different analytical methods are in close agreement and show that the S

enantiomer forms a stronger complex with γ-CD than does its R antipode. Separate injections of the enantiomers in FSCE (section 4.4.1.) showed that the R enantiomer had a faster migration time than the S isomer i.e. its interaction with γ-CD was lower and consequently it migrated fastest. Injections of the enantiomers in HPLC showed that the R enantiomer had a larger k′ value than the S isomer, which indicates that the R enantiomer was more strongly retained by the chromatographic system and so formed a weaker complex with γ-CD. These results are in accordance with the relative strength of K_f values calculated above. The optimum concentration of γ-CD for chiral resolution of mequitamium in FSCE was determined to be 5.0 mM (section 4.4.3.) while in HPLC (section 3.4.3.) 12 mM was found to be optimal.

Plots of k'_{obs} versus $(k'_s - k'_{obs})/(CD)$ in section 3.4.3. showed a linear relationship, which according to Allenmark 1991, is indicative of 1:1 host-guest complex stoichiometry. Subsequent NMR Job experiments and Foster-Fyfe plots (see section 5.4.6.) also showed that a 1:1 complex was formed with a racemic K_f of 333 M^{-1}. $k'_{s.cd}$. values of the R and S enantiomers in section 3.4.3. suggested that resolution of the antipodes in HPLC was influenced by significant retention of analyte:γ-CD complexes on the stationary phase surface. FSCE experiments did not involve such interaction with a stationary phase. Therefore the higher enantio-resolution values generated with FSCE seem to arise as a consequence of the very high efficiency of the system allowing separation based solely on relatively small differences in the enantiomeric K_f values which produce differences in migration times of the resulting diastereoisomeric complexes. Chiral resolution values of mequitamium were significantly higher with all three CD types in FSCE than in HPLC and only 5.0 mM of γ-CD was required to produce the highest chiral resolution value in FSCE whereas HPLC required a γ-CD concentration of 12 mM. As stated in section 6.1.3. the discrepancy between HPLC and FSCE analyte separation abilities and the need for lower concentrations of γ-CD in FSCE may be related to the HPLC system having only 2500 - 4500 theoretical plates, whilst the FSCE column routinely produced values > 200,000. All these results concerning the interaction between γ-CD and mequitamium are in good agreement with one another and provide a strong insight

into the strength and nature of the inclusion complex formed by the two interacting species.

In summary, FSCE generally proved to be a better analytical technique for generating enantio-resolution values for the various analytes than did HPLC. All of the phenethylamines, four of the five examined propranolol analogues and the enantiomers of mequitamium were best separated using FSCE. This was linked to the much higher number of theoretical plates in FSCE enabling higher enantio-resolution values. The CDs responsible for the maximum observed chiral resolution values in each analytical technique were seen to vary for the phenethylamines and the propranolol analogues. For mequitamium, γ-CD provided the highest chiral resolution in both HPLC and FSCE. However, the four different modes of HPLC examined in this work allowed a more flexible approach to the problem of chiral separations e.g. peracetyl-β-CD proved impractical to utilise in FSCE but in HPLC experiments with a PGC surface, novel chiral resolutions for two propranolol analogues were achieved within very short analysis times. In practical terms, CDs in short supply can be more efficiently employed in FSCE which requires much small amounts of buffer and hence buffer additives than HPLC.

The influence of CD concentration, pH, buffer and solvent additives in HPLC and FSCE was also examined. Small changes in these parameters were shown to be capable of strongly influencing the measured enantio-resolution values.

6.3. Comparison of NMR, FSCE and HPLC.

Analyte-CD interactions in FSCE experimental conditions may be more comparable with NMR experimental data than HPLC, as the host and guest molecules in both FSCE and NMR interact with each other purely in solution without the involvement of a solid surface, as in HPLC. The nature of the HPLC stationary phase, whether chiral or even achiral (Walhagen and Edholm 1991), can play an important role in

chiral separations. Therefore to investigate analyte-CD interactions it could be more prudent to compare FSCE and NMR results.

Sections 5.1.2. - 5.1.6. include many references of how NMR chemical shifts of host and guest molecules, often related to other analytical data e.g. HPLC, GC and FSCE, may be employed to more fully understand the complexation process involving CDs in aqueous media. In the following sections K_f values of the analytes, determined by NMR, HPLC or FSCE, are used to estimate the relative strengths of complexation between the analytes and various CDs. Along with NMR measured chemical shifts and stoichiometry ratios, HPLC k′ values and FSCE migration times, it is hoped to show whether meaningfull comparisons can be drawn between the analytical data generated in each technique. Molecular modelling results of β- and Ac-β-CD, given in section 5.2.4., are also alluded to with the purpose of providing further insights into the CD inclusion complexation process.

6.3.1. Phenethylamines - a Comparison of FSCE, HPLC and NMR.

β- and Ac-β-CD were shown to cause enantio-resolution of all phenethylamines, except noradrenaline and isoprenaline, when using FSCE and/or HPLC (see sections 4.2. and 3.2 respectively). Peracetyl-β-CD also resolved the enantiomers of ephedrine, oxilofrine and orciprenaline in conjunction with a PGC column. However, complementry NMR experiments using peracetyl-β-CD were not successful. $CDCl_3$ was required for the dissolution of this hydrophobic CD derivative and the resulting chemical shifts of the analytes were all very low, ≤ 0.002 ppm, probably due to the highly non-polar environment preventing significant analyte-CD interaction. It was not possible then to use NMR to probe the analyte-CD interaction in this instance.

Other CDs examined e.g. α- and γ-CD, did not induce significant chemical shift changes of the analytes nor did they show evidence of analyte inclusion themselves by virtue of insignificant chemical shifts of their inner H3" and H5" protons. Neither

α-, HP-α- nor γ-CD markedly altered the migration times of the analytes in FSCE or HPLC experiments. In conjunction with the NMR data this indicates that these CDs interact weakly, if at all, with the phenethylamine analogues. The small size of the α-CD cavity, 5.7 Å and the much larger size of the γ-CD cavity, 9.5 Å (see Table 1.2., p.10), would not be sterically favourable for a 'tight-fit' of the analytes, precluding their ability to elicit a stereo-specific inclusion interaction.

Only β- and Ac-β-CD, which had provided analyte chiral resolution in FSCE, were found to show significant analyte chemical shifts in the NMR experiments. β-CD caused a larger shift in the aromatic region of the analytes than Ac-β-CD, but Ac-β-CD was the only CD to produce analyte signal splitting for all the phenethylamines, except noradrenaline and salbutamol. The inner H3" and H5" protons of β-CD experienced strong upfield shifts (shielded) from all the phenethylamines, while the equivalent protons in Ac-β-CD showed weaker shielding. These results suggest that the strength of analyte inclusion is greatest in the presence of β-CD but that Ac-β-CD was the better chiral discriminating agent of the two CDs. In FSCE the highest R_s were obtained with Ac-β-CD but the migration times of the analytes were always faster than with β-CD i.e. analyte complexation was weaker with Ac-β-CD than β-CD (by virtue of the faster migration times with Ac-β-CD) but the chiral resolution values were greater. Therefore, the strength of binding and chiral discrimination of each CD towards the phenethylamines, based on relative CD chemical shift strengths and analyte signal splittings in NMR, may be related to the chiral resolution values and relative migration time results of the FSCE experiments.

Resolution in FSCE depends on the free analyte having a different apparent electrophoretic mobility than its complexed form *and* on the formation constants, K_f, for each enantiomer-CD complex being different (Wren and Rowe 1992). In NMR signal splitting may arise as a result of differences in K_f values and / or intrinsic chemical shift differences of the complexes (Uekama *et al.* 1985 and Cooper *et al.* 1991). If, as above, certain analytes such as ephedrine are chirally resolved in FSCE (indicating differences in K_f values), but no signal splitting is observed in NMR

under the same conditions, it appears that the absence of enantiomeric separation in NMR is due mainly to the lack of intrinsic magnetic inequivalence differences in each enantiomer-CD complex.

Attempts were made to measure the K_f values of etilefrine with β- and Ac-β-CD (section 4.2.4., p.104) by FSCE. Although the actual K_f values could not be measured with Ac-β-CD due to its low solubility, it was found that etilefrine formed a weaker complex with Ac-β-CD than β-CD. This result also agrees that the NMR evidence that the proposed strength of analyte interaction is greatest with β-CD. NMR data for the measurement of K_f values proved inconclusive. Foster-Fyfe plots of etilefrine with β- and Ac-β-CD were not linear (section 5.2.4.) so it proved impossible to obtain a K_f value. The reason for this is unclear. However it has been shown here that stronger analyte binding to a given CD does not guarantee greater chiral discrimination. The stoichiometry of the etilefrine:β- and Ac-β-CD complexes was measured and both were found to be 1:1 (see Fig. 5.6., p.155). It is presumed that the other phenethylamines form complexes of the same stoichiometry as they all possess only one aromatic ring, the main site of inclusion.

Compounds with R_2 = methyl i.e. ephedrine and oxilofrine, showed a large shift of their H1 resonances with β- and Ac-β-CD, but the chiral centre of each analyte, the H1 proton signal, was split only by Ac-β-CD. Differences in hydrogen bonding interactions between each CD and the guest side chains or conformational rearrangement of the guest side-chains were thought likely to have been influential in the observed differences in analyte side-chain signal splitting and chemical shifts. The positioning of the 2 and 3 acetyl groups of Ac-β-CD would allow them to act as hydrogen bond acceptors with analyte NH or OH groups in a manner which would not be possible for the underivatised hydroxyl groups of β-CD.

However relative to H3" / H5", the CD H6" signals were more strongly shifted in AC-β-CD than β-CD indicating that the CH_2 protons at the primary end of the Ac-β-CD torus were significantly involved in the analyte-CD interaction mechanism.

Molecular modelling (section 5.2.4.) showed that the secondary end of Ac-β-CD had an increased width compared to that of β-CD. Such conformational changes were thought to occur only with *per*-derivatised CDs (Harata *et al.* 1984 and Ventura *et al.* 1994). The increased width of the Ac-β-CD cavity could have allowed the analytes a deeper insertion, which in turn may have altered the chemical shift of the H6" protons located towards the primary end of the CD. Rekharsky *et al.* 1994 stated that deeper penetration of guest molecules may account for observed changes in CD H6" signals. Furthermore, Dodziuk *et al.* 1994 found that high K_f values of 1,2,3 tri-*t*-butylnaphthalene with γ-CD were accompanied by a shallow insertion of the guest into the CD cavity. In this case, it appears that lower K_f values of the phenethylamines with Ac-β-CD occur with a deeper insertion into the CD cavity. The strength of analyte-CD binding cannot then be solely linked to the depth of analyte insertion within the CD cavity.

HPLC studies showed that only three of the phenethylamines could be chirally resolved using either a β-CD containing mobile phase with a PGC column or a bonded β-CD column (see Table 6.1). In NMR, all the analytes showed chemical shift changes of their aromatic and side-chain protons in the presence of β-CD and five of them gave signal splitting. Brown *et al.* 1993 noted that thermodynamic differences that may allow chiral detection were not always large enough to produce enantiomeric resolution in NMR. Cooper 1991 found no clear correlation in chiral resolution between NMR and HPLC (which relies on thermodynamic differences for separation) with a series of pharmaceutical compounds when examined with β-CD. Therefore sufficient thermodynamic differences that may allow detection of chiral complexation by NMR spectroscopy, where the energy differences involved in distinction are small, may not necessarily be sufficient to induce chromatographic separation of the complexes. This would help explain the poor NMR/HPLC correlation found here with β-CD and the phenethylamines. Isoprenaline was the only phenethylamine to show NMR signal splitting in the presence of Ac-β-CD which was not subsequently resolved by FSCE experiments with Ac-β-CD. There was clearly a closer correlation between NMR signal splitting and chiral resolution ability in

FSCE than with HPLC. FSCE appears to have been better suited than HPLC to enable small thermodynamic differences in analyte-CD interactions to be translated into observable chiral resolutions, probably due in part to the much greater separation efficiencies of FSCE noted throughout this work.

6.3.2. Propranolol Analogues - Comparison of FSCE, HPLC and NMR.

These compounds were generally well resolved by HPLC and FSCE (sections 3.3. and 4.3. respectively) using β-CD, He-β-CD, Me-β-CD and to a lesser extent Ac-β-CD and peracetyl-β-CD. Under similar conditions to those present in the FSCE studies, NMR experiments utilised these CDs which had proven capable of resolving the propranolol analogues. Although compound **6** was not resolved by HPLC and proved impractical to examine by FSCE, it was still studied in the presence of the various CDs using NMR spectroscopy.

Peracetyl-β-CD did not show any marked analyte chemical shifts in the NMR experiments even though it had resolved the enantiomers of compounds **4** and **5** by HPLC. Once again however, it was necessary to use the highly non-polar solvent $CDCl_3$ to allow dissolution of a sufficient amount of peracetyl-β-CD to examine by NMR. As stated before, the resulting non-polar environment was likely to have been the cause of the very small chemical shifts then observed.

In the presence of β-CD all the propranolol analogues showed downfield shifts of their aromatic protons indicative of inclusion with the CD, which agrees in part with the findings of Greatbanks and Pickford 1987 who examined propranolol with β-CD. The alkyl side-chains of the analytes were strongly deshielded and signal splitting was observed at the methylene group in the 3 position for compounds **1-3** and at the terminal R group for compounds **3** and **6** (see Table 5.7. on p.158). In FSCE experiments, upon which the NMR solvent conditions were based, enantio-resolution values were in the order compound **2>1**, **5>4** and **1>4**, **2>5** i.e. a *t*-butyl group

in the R position promoted chiral resolution as did the presence of a naphthyl ring. It was also noted that on average the NMR chemical shifts of the naphthyl protons of compounds **1-3** were generally greater than those of the phenyl protons of compounds **4** and **5**, suggesting a stronger complexation. Related to this was the FSCE migration times of those compounds with a naphthyl group i.e. compounds **1-3**, which were longer than those of compounds **4** and **5**, with R′ = phenyl. This again indicates that these analytes were interacting more strongly with β-CD. There is thus concurrancy between the size of analyte aromatic chemical shifts, the migration times of the analytes in FSCE and their resulting enantio-discrimination.

The H3" protons of β-CD were more strongly shielded than the H5" protons suggesting a more shallow insertion of all the analytes into the β-CD cavity. H1", H2" and H4" protons located outside the CD cavity were more strongly shifted than the H6" protons, indicating that analyte interaction is not significant at the narrow primary end of the torus.

A *t*-butyl R group promoted chiral resolution in both FSCE and HPLC (using a bonded β-CD column) with the resolution of compound **2>1** and **5>4**. The presence of a naphthyl group on the analytes did likewise i.e. resolution of compound **1>4** and **2>5**. Analyte migration times and HPLC k′ values followed the same pattern in the presence of β-CD, whereby longer analysis times were directly related to the analytes having either a naphthyl ring or a *t*-butyl group i.e. compound **1>2**, **5>4** and **1>4**, **2>5**. Therefore increases to the size and hydrophobicity of the analytes caused increased complexation with β-CD, directed towards the wider secondary end of β-CD, which in turn promoted chiral recognition.

Ac-β-CD produced some of the lowest analyte NMR chemical shifts of all the CDs examined. There was little difference between the chemical shift values obtained with each analyte and no signal splitting was recorded. The H5" protons of Ac-β-CD were less strongly shielded than the H3" protons with all the analytes, which suggests a shallow insertion at the secondary rim. However, the externally positioned hydrogen

nuclei, H1", H2" and H4", generally displayed stronger shielding effects than the acetyl groups on the secondary rim of the CD. Low FSCE chiral resolution values of the propranolol analogues were also found with this CD. These analytes appear to be interacting with the periphery of Ac-β-CD in a poorly stereogenic manner, hence their related pattern of weak chemical shifts, absence of signal splitting and low FSCE chiral resolution.

The overall magnitude of the aromatic and alkyl chemical shifts of compounds **1-6** were greatest in the presence of Me-β-CD. The chiral proton in position 2 on the alkyl side-chain was more strongly shifted for compounds **4** and **5** (R′ = phenyl) than with the other analytes (R′ = naphthyl). Furthermore, it was found in FSCE experiments with Me-β-CD that compounds **4** and **5** tended to be chirally resolved to a greater extent than the other analogues. All the analyte signals in NMR were shifted upfield by Me-β-CD but no signal splitting was observed for any of the compounds.

The resolution in FSCE was adversely affected by the presence of a naphthyl group, with the enantiomeric separation of compound **4>1** and **5>2**. These results are exactly opposite in trend to those observed with β-CD (see Table 4.6.), where a naphthyl group increased analyte resolution i.e. compound **1>4** and **2>5**. A *t*-butyl R group was seen to enhance chiral resolution in the order compound **2>1** and **5>4**, which is in agreement with the trend observed using β-CD. These results demonstrate clearly how derivatisation of the CD rim can lead to differences in chiral selectivity. The large and bulky naphthyl group of compounds **1**, **2** and **3** seems to have been sterically less favourable for chiral discrimination with the derivatised Me-β-CD macrocycle, whereas a *t*-butyl R group was beneficial for separation. Strong chemical shifts of the R groups in NMR support the FSCE observation that these groups were influential in the chiral recognition process.

The Me-β-CD H3" signal was more strongly shifted than the H5" signal only by compounds **4** and **5** (see Table 5.14.), which suggests these compounds had a shallower insertion into the CD cavity than the other analogues. FSCE experiments

had shown these two analogues to be well resolved by Me-β-CD within relatively quick analysis times when compared to the other analytes. A subsequent measurement of each analyte's K_f value with Me-β-CD in FSCE (section 4.3.6.) showed they complexed in the order - compound **2>1>3>5>4.** Weaker binding to the CD of compounds **4** and **5**, coupled with their relatively more shallow inclusion into the CD, did not cause lower chiral resolution values in FSCE but infact produced an increase in enantio-resolution. The order of K_f values exactly matches the order of analyte hydrophobicity indicating that hydrophobicity is a major factor in complex formation but not necessarily of chiral resolution. As pointed out with the phenethylamines and Ac-β-CD in section 6.3.1., the strength of analyte-CD binding cannot be solely linked to the depth of analyte insertion within the CD cavity. Changes to the CD rim or cavity dimensions as a result of derivatisation of the hydroxyl groups, may also be important as demonstrated e.g. see section 5.2.4., where the width of the CD cavity was seen to vary from β- to Ac-β-CD.

No chiral resolution was found for any of the propranolol analogues in HPLC when using Me-β-CD as a mobile phase additive with either a C8 or PGC column. Strong decreases in k′ value of all the analytes, except compound **6**, were noted in the presence of the CD. Analyte-CD complexation may have been occurring in solution or the formation of a 'dynamic stationary phase' on the column surface may have been responsible for the observed changes. Any comparison with the results of FSCE and NMR was therefore not meaningful.

All the analytes showed chemical shifts of their aromatic and alkyl protons in the presence of HE-β-CD. Compounds **2, 3, 5** and **6** showed clear signal splitting in either their side-chains positions 1, 3 or their R groups, which shows that this portion of each analyte was in close proximity to the CD chiral environment. The H3" and H5" protons of HE-β-CD were strongly shielded in the presence of compounds **1-6** and the hydroxyethyl signals were more strongly shifted than the H6" protons at the primary end of the torus, indicating that analyte interaction was occurring at the wider secondary opening of the CD. The FSCE resolution values of the analytes were

higher with this CD derivative than with any of the other examined CDs. The chiral separations were in the order - compound **4>1** (the trend same as with Me-β-CD but opposite to β-CD) and compound **2>5** (the opposite result to Me-β-CD but similar to β-CD). K_f values were higher with HE-β-CD than Me-β-CD indicating stronger complexation. The relative K_f values of the propranolol analogues in FSCE were found to be in the order - **2>1>3>5>4**, which is identical to that found with Me-β-CD. The migration times of compounds **1-5** were also longer upon the addition of HE-β-CD than any of the other CDs, which itself may suggest greater complexation between the analogues and this CD. As stated before however, changes in the nature of the groups on the CD cavity can result in selectivity differences between the CDs and the strength of analyte-CD complexation does not necessarily equate to greater chiral resolution.

HE-β-CD was the only CD derivative to resolve the enantiomers of one of the propranolol analogues by HPLC, namely compound **2**, which also had the largest K_f value in the presence of HE-β-CD and the greatest chiral resolution value (R_s = 1.67) in FSCE experiments. The low effeciency of the chromatographic system was probably the reason why this technique only provided chiral resolution for that analyte which had been proven to undergo the greatest chiral discriminating action with HE-β-CD.

6.3.3. Mequitamium - a Comparison of FSCE, HPLC and NMR.

From amongst the ten different CDs examined with mequitamium in NMR, the magnitude of analyte chemical shifts were noted to be in the order γ-CD> HP-β-CD> HP-γ-CD > β-CD > α-CD ≈ HP-α-CD. This was especially true in the case of the aromatic protons of the tricyclic group. These protons were most strongly shielded by γ-CD indicating significant inclusion complexation. Three CDs were able to produce enantio-resolution of mequitamium in FSCE in the following order, γ-> HP-β- >HP-γ-CD. Analyte migration times in FSCE with these three CDs were

found to increase as follows γ-CD < HP-γ-CD < HP-β-CD. Therefore high enantio-discrimination in FSCE was not always accompanied by longer analyte migration times (suggesting greater analyte-CD interaction), although they did correlate better with chemical shift strengths in NMR, where the order was found to be γ-CD > HP-β-CD > HP-γ-CD.

The stoichiometry of the complexes formed by mequitamium with both γ-CD and HP-β-CD (section 5.4.6.) were found to be 1:1 via NMR generated Foster-Fyfe plots and HPLC plots (section 3.4.3.) of k'_{obs} vs $(k'_s - k'_{obs})/(CD)$, measured in the case of γ-CD only. Of the two mequitamium enantiomers, the R antipode was seen to have the fastest migration times in FSCE and the longest k′ value in reversed phase HPLC, when examined in the presence of γ-CD. Both results show that this enantiomer formed a weaker complex with the CD than did the S isomer. The magnitude of K_f values obtained with γ-CD for each enantiomer in both FSCE and HPLC (see section 6.2.3.) were found to be in close agreement and proved that the R isomer was interacting more weakly with γ-CD in each technique, accounting for the value of its migration times and k′ values relative to those of the S enantiomer. The NMR measured K_f value of mequitamium with HP-β-CD was found to be much lower than that found with γ-CD, although its FSCE migration time in the presence of HP-β-CD was longer. Similarly the HPLC k′ value of mequitamium with HP-β-CD (6.75, 7.44) was lower than that of γ-CD (18.7, 20.7) but chiral resolution was lower (R_s = 0.3 compared to R_s = 1.06 with γ-CD). Therefore an inverse relationship between NMR measured K_f values and both FSCE and HPLC analysis times of mequitamium in the presence of HP-β-CD was observed. This is in direct contrast to the aforementioned relationship found with these techniques using γ-CD.

If the enantiomers possessed sufficiently different K_f values to allow their resolution in FSCE and HPLC, it follows that the absence of enantiomeric signal splitting in NMR was probably caused by the absence of intrinsic differences in the magnetic environments of the enantiomer-CD complexes. This phenomenon was also observed with some of the phenethylamines e.g. ephedrine, in the presence of Ac-β-CD.

In summary, certain trends were noted between the analytical techniques when examining the same chiral analytes. There was no continuous direct relationship between K_f values, FSCE migration times / chiral resolution values and NMR generated chemical shifts / signal splitting of the analytes.

With the phenethylamines, lower K_f values were associated with higher FSCE resolution, weaker analyte chemical shifts, and the absence of NMR signal splitting when comparing the results obtained with β- and Ac-β-CD. With the propranolol analogues no consistant pattern could be established between these parameters when examining a range of CDs. Increased FSCE chiral resolution and analyte migration times were not always complemented by greater NMR chemical shift strengths or signal splitting e.g. Me-β-CD produced lower FSCE resolutions than HE-β-CD but overall analyte chemical shift values were greater, K_f values were lower and migration times were faster. The results with HE-β-CD did show a good comparison, with increased chemical shifts and signal splitting accompanied by greater FSCE resolution and longer migration times. With Ac-β-CD chemical shifts were low, there was no signal splitting and chiral resolution was poor. When using β-CD increased analyte aromatic shifts were matched with longer migration times and higher enantio-resolutions in FSCE. The trend in results with mequitamium, like those of the other analytes, was found to depend on the CD in use. γ-CD and HP-β-CD produced contrasting results in their relationship between K_f values and migration times in FSCE.

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